

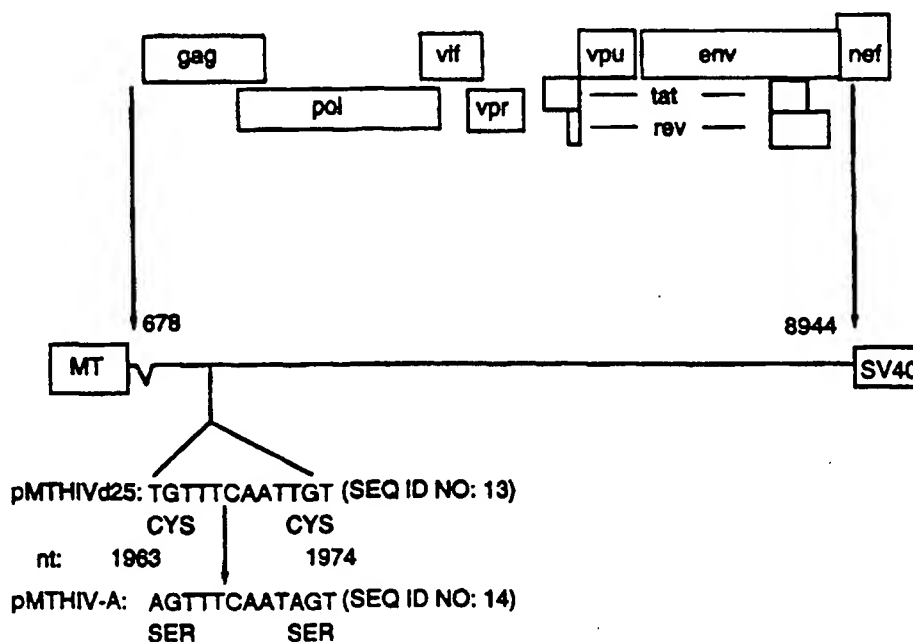


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(71) Applicant (for all designated States except US): CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Avenue West, Willowdale, Ontario M2R 3T4 (CA).			
(72) Inventors; and (75) Inventors/Applicants (for US only): ROVINSKI, Benjamin [CA/CA]; 70 Winding Lane; Thornhill, Ontario L4J 5H6 (CA). CAO, Shi-Xian [CA/CA]; Apartment #408, 716 The West Mall, Etobicoke, Ontario M9C 4X6 (CA). YAO, Fei-Long [CN/CA]; 81 Elsa Vineway, North York, Ontario M2J 4H8 (CA). PERSSON, Roy [CA/CA]; 7 Bishop Avenue, Unit 604, North York, Ontario M2M 4J4 (CA). KLEIN,			

(54) Title: RETROVIRUS-LIKE PARTICLES MADE NON-INFECTIONOUS BY A PLURALITY OF MUTATIONS



## (57) Abstract

Non-infectious, retrovirus-like particles contain mutations to reduce gag-dependent RNA-packaging of the gag gene product, eliminate reverse transcriptase activity of the pol gene product, eliminate integrase activity of the pol gene product and eliminate RNase H activity of the pol gene product through genetic manipulation of the gag and pol genes. The corresponding nucleic acid molecules are described. The non-infectious, retrovirus-like particles have utility in *in vivo* administration including to humans and in diagnosis.

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TITLE OF THE INVENTION  
RETROVIRUS-LIKE PARTICLES MADE  
NON-INFECTIOUS BY A PLURALITY OF MUTATIONS

5                   FIELD OF THE INVENTION

The present invention relates to the field of immunology and is particularly concerned with retrovirus-like particles (sometimes termed pseudovirions), made non-infectious by a plurality of mutations.

10                   REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of co-pending United States Patent Application No. 08/292,967 filed August 22, 1994.

15                   BACKGROUND OF THE INVENTION

Human immunodeficiency virus is a human retrovirus and is the etiological agent of acquired immunodeficiency syndrome (AIDS). Since AIDS was first reported in the US in 1981, more than 194,000 people have died of AIDS and over 330,000 cases of HIV infection have been reported in the U.S. alone. Worldwide, it is estimated that more than 17 million people have been infected with HIV.

More than 100 AIDS-related medicines are in human clinical trials or awaiting FDA approval but there is currently no cure for the disease.

25                   There is, therefore, a clear need for immunogenic preparations useful as vaccine candidates, as antigens in diagnostic assays and kits and for the generation of immunological reagents for diagnosis of HIV and other retroviral disease and infection.

30                   Particular prior art immunogenic preparations include non-infectious, non-replicating HIV-like particles. Thus PCT applications WO 93/20220 published October 14, 1993 and WO 91/05860 published May 2, 1990 (Whitehead Institute for Biomedical Research), teach  
35                   constructs comprising HIV genomes having an alteration in a nucleotide sequence which is critical for genomic RNA packaging, and the production of non-infectious

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immunogenic HIV particles produced by expression of these constructs in mammalian cells.

PCT application WO 91/07425 published May 30, 1991 (Oncogen Limited Partnership) teaches non-replicating  
5 retroviral particles produced by co-expression of mature retroviral core and envelope structural proteins, such that the expressed retroviral proteins assemble into budding retroviral particles. A particular non-replicating HIV-1 like particle was made by coinfecting  
10 mammalian host cells with a recombinant vaccinia virus carrying the HIV-1 gag and protease genes and a recombinant vaccinia virus carrying the HIV-1 env gene.

In published PCT application WO 91/05864 in the name of the assignee hereof (which is incorporated herein by  
15 reference thereto), there are described particular non-infectious, non-replicating retrovirus-like particles containing at least gag, pol and env proteins in their natural conformation and encoded by a modified retroviral genome deficient in long terminal repeats and containing  
20 gag, pol and env genes in their natural genomic arrangement.

Virions of HIV comprise two copies of the single-stranded RNA genome enclosed within a capsid. After penetration into a susceptible host cell, the HIV genome  
25 is copied by the viral reverse transcriptase into single-stranded DNA that is thought to be translocated into the nucleus, wherein a cellular DNA polymerase synthesizes the second DNA strand. The double-stranded copy is then integrated, at random, into one of the host chromosomes,  
30 resulting in a duplication of a region of the viral genome at the extremities of the genome. The long-terminal repeat (LTR) of the integrated provirus is recognized by a cellular RNA polymerase and the transcribed RNA is translated to give rise to viral  
35 proteins. The RNA transcripts can also be packaged into new virions that leave the cell by a process of budding.

The HIV genome encodes at least nine different proteins. The three major genes, gag, pol and env are common to all retroviruses and encode virion proteins.

The differential expression of these genes is achieved through a complex pattern of processing of the primary precursor transcript. Only the GAG and POL proteins are produced from the unspliced mRNA corresponding to the genomic RNA of the virion. The ENV protein is translated from a mRNA species that has undergone a single splicing event to delete the gag and pol coding sequences, and other proteins are produced from mRNA species that are spliced several times. The general structure of HIV is reviewed by Kieny et al (ref. 8).

Thus, it may be advantageous under particular circumstances to produce retrovirus-like particles (and in particular HIV-like particles) by mutating other portions of the HIV genome contributing to infectivity and replication of the virus. Such modifications may be modifications of the gag and pol gene products.

There is currently no vaccine nor effective treatment for AIDS. Heat-inactivated anti-HIV antiserum obtained from HIV-infected people and inactivated HIV are currently commercially available as components of many diagnostic methods. For safety, ease of handling, shipping, storage and use, it may be preferable to replace such antigen and heat-inactivated antisera by non-infectious HIV-like particles and antisera generated by immunization with non-infectious HIV-like particles as described above and particularly in WO 91/05864. Furthermore, antisera generated by immunization with these non-infectious HIV particles do not require heat inactivation to remove infectious HIV. The HIV-like particles described in WO 91/05864 are entirely deficient in replication and infection. However, because of the seriousness of HIV infection, it may be desirable under

certain circumstances to provide retrovirus-like particles deficient in a plurality of elements required for infectivity and/or replication of HIV but dispensable for virus-like particle formation. Furthermore, since  
5 prior art HIV-like particles contain many of the HIV proteins in substantially their natural conformations, a host immunized therewith may mount an immune response immunologically indistinguishable from infection by HIV and it may be desirable to be able to distinguish between  
10 inactivated HIV and non-infectious, non-replicating HIV particles and antisera generated by virulent HIV and non-infectious, non-replicating HIV-like particles. Thus, in the development of AIDS vaccine candidates, immunogenic preparations and diagnostic methods and kits, it would be  
15 useful to provide an HIV-like particle deficient in a plurality of elements required for infectivity and/or replication and optionally immunologically or otherwise distinguishable from virulent HIV.

#### SUMMARY OF THE INVENTION

20 The present invention is directed towards the provision of retrovirus-like particles made non-infectious by a plurality of mutations.

Accordingly, in one aspect of the invention there is provided a non-infectious immunogenic, retrovirus-like  
25 particle comprising, in an assembly, gag, pol and env gene products, wherein at least one modification has been made to the pol and/or gag gene product, to effect at least one of the following :

(a) reduce gag-dependent RNA packaging of the gag  
30 gene product;

(b) substantially eliminate reverse transcriptase activity of the pol gene product;

(c) substantially eliminate integrase activity of the pol gene product; and

35 (d) substantially eliminate RNase H activity of the pol gene product.

The reduction in gag dependent RNA packaging may be effected by replacing or deleting at least one amino acid residue contributing to gag-dependent RNA packaging in the gag gene product. In an illustrative embodiment, the at least one amino acid may be contained within amino acids Cys<sup>392</sup> to Cys<sup>395</sup> of the gag gene product of HIV-1 LAI isolate or the corresponding region of other retroviral gag gene products and Cys<sup>392</sup> and/or Cys<sup>395</sup> or both cysteines may be replaced by serine residues.

10 In one specific illustrative embodiment of the invention, the substantial elimination of reverse transcriptase activity of the pol gene product may be effected by deletion of at least a portion thereof contributing to reverse transcriptase activity. The at  
15 least a portion of the pol gene product may be contained between amino acids Pro<sup>168</sup> and Leu<sup>777</sup> of the pol gene product of HIV-1 LAI isolate or the corresponding region of other retroviral pol gene products. The substantial elimination of integrase activity of the pol gene product  
20 may be effected by deletion of at least a portion thereof contributing to integrase activity and the at least a portion of the pol gene product may be contained between amino acids Phe<sup>728</sup> and Asp<sup>1016</sup> of the pol gene product of HIV-LAI isolate or the corresponding region of other  
25 retroviral pol gene products.

The substantial elimination of RNase H activity of the pol gene product may be effected by deletion of at least a portion thereof contributing to RNase H activity.

In a particular embodiment of this aspect of the  
30 invention substantial elimination of reverse transcriptase, integrase and RNase H activities may be simultaneously effected by deleting a portion of the pol gene product corresponding to amino acids Pro<sup>192</sup> to Trp<sup>835</sup> of HIV-1 LAI isolate, or the corresponding region of  
35 other retroviral pol gene products.

In a further aspect of the invention, the non-infectious retrovirus-like particles of the invention may additionally comprise at least one non-retroviral antigenic marker. The incorporation of antigenic markers into non-infectious retrovirus-like particles is described in our copending U.S. patent application No. 08/290,105 filed August 25, 1994, the disclosure of which is incorporated herein by reference. The at least one antigenic marker may be contained within the gag gene product to form a hybrid gag gene product having the particle-forming characteristics of unmodified gag gene product. In a particular embodiment, the at least one antigenic marker may be inserted into an insertion site of the gag gene product at an antigenically-active insertion site and the insertion site may be located between amino acid residues 210 and 211 of the gag gene product of the HIV-1 LAI isolate or the corresponding location of other retroviral gag gene products. The at least one antigenic marker may comprise from 1 to 4 tandem copies of the amino acid sequence AFDTRNRIIEVEN (SEQ ID NO: 1) or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence AFDTRNRIIEVEN.

The marker sequence also may be provided by deleting or preventing production of an amino acid sequence that corresponds to an epitope of a retroviral protein. Such epitope may comprise an immunodominant region of gp41, which provides endogenous anchoring function. When such endogenous anchoring function is removed in this way, the anchoring function is provided by a different antigenic anchor sequence.

In a further particular embodiment of this aspect of the invention, the env gene product of the retrovirus-like particles as provided herein may be a modified env gene product in which endogenous anchoring function has been replaced by a different antigenic anchor sequence



operatively connected to the env gene product to anchor the env gene product to the retrovirus-like particle and the anchor sequence may be inserted into an insertion site of the env gene product adjacent to and upstream of functional cleavage sites of the env gene product. The insertion site may be located between amino acid residues 507 and 508 of the env gene product of the HIV-1 LAI isolate or the corresponding location of other retroviral env gene products. The anchor sequence may include an amino acid sequence WILWISFAISCFLLCVLLGFIMW (SEQ ID NO: 2) or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence WILWISFAISCFLLCV LLGFIMW.

In yet another embodiment, the anchor sequence may include an amino acid sequence STVASSLALAIMIAGLSFWMCNSGSLQ (SEQ ID NO: 3) or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence STVASSLALAIMIAGLSFWMCNSGSLQ.

In another embodiment, the anchor sequence may include an amino acid sequence WILWISFAISCFLLCVVCWGSSCGPAKKATLGATFAFDSKEEWCREEKKEQWE (SEQ ID NO: 4) or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence WILWISFAISCFLLCVVCWGSSCGPAKKATLGATFAFDSKEEWCREEKKEQWE.

Alternatively, the immunodominant region of the env gene product may be modified by substitution or removal of at least one amino acid therefrom to substantially prevent recognition of the immunodominant region in the resulting mutation by sera from retrovirus-infected hosts.

In a particular embodiment of the latter alternative, the retrovirus may be HIV-1 and the immunodominant region may contain the amino acid sequence LGIWGCSGKLIC (SEQ ID No: 27). Specific amino acid sequences of mutations which may be provided herein

include LGIWGCTGRLIC (SEQ ID No: 28), LGIWGCAFR LIC (SEQ ID No: 29) and LGIWGCTLELIC (SEQ ID No: 30).

The retrovirus-like particle generally is a human retrovirus-like particle, particularly derived from HIV-1, HIV-2, HTLV-1 or HTLV-2. Specifically, the human retrovirus may be HIV-1 and the env gene product may be an LAI env gene product, an MN env gene product, an env gene product from a primary HIV-1 isolate, or an env gene product antigenically equivalent thereto. The gag and pol gene products may be derived from an HIV-1 isolate different from the HIV-1 isolate from which the env gene product is derived. In particular, in such particles, the env gene product may be derived from a primary HIV-1 isolate.

The present invention also includes nucleic acid molecules encoding the non-infectious, retrovirus-like particles of the invention. Accordingly, in another aspect of the invention, there is provided a nucleic acid molecule encoding a non-infectious, immunogenic, retrovirus-like particle, comprising a modified retroviral genome deficient in long terminal repeats and containing gag, pol and env genes in their natural genomic arrangement and means for expression operatively connected to the modified retroviral genome for production of gene products in cells to produce non-infectious, immunogenic, retrovirus-like particles comprising an assembly of gag, pol and env gene products, wherein at least one codon in the gag or pol gene has been mutated to effect at least one of the following:

- (a) reduce gag-dependent RNA packaging activity of the gag gene product;
- (b) substantially eliminate reverse transcriptase activity of the pol gene product;
- (c) substantially eliminate integrase activity of the pol gene product; and

(d) substantially eliminate RNase H activity of the pol gene product. The nucleic acid molecule may comprise a DNA molecule containing the characteristic genetic elements present in a SacI 678 to XhoI 8944 fragment of the genome of the HIV-1 LAI isolate. The modified genome also may be deficient in primer binding site and/or an RNA packaging signal.

The reduction of gag-dependent RNA packaging may be effected by mutagenesis of a region thereof encoding at least one amino acid contained within a region of the gag gene product corresponding to Cys<sup>392</sup> to Cys<sup>395</sup> of the HIV-1 LAI isolate, or the corresponding region of other retroviral gene products, and Cys<sup>392</sup> and/or Cys<sup>395</sup> or both cysteines may be replaced by serine residues.

In one specific illustrative embodiment of the invention, the substantial elimination of reverse transcriptase activity of the pol gene product may be effected by deletion of at least a part of the pol gene encoding reverse transcriptase and the at least a part of the pol gene deleted may be contained between nucleotides 2586 and 4265 of the pol gene of HIV-1 isolate LAI or the corresponding region of other retroviral pol genes.

In an additional aspect, the substantial elimination of integrase activity of the pol gene product may be effected by deletion of at least a part of the pol gene encoding integrase and in an illustrative embodiment the at least a part of the pol gene deleted may be contained between nucleotides 4266 and 5129 of the pol gene of HIV-1 isolate LAI or the corresponding region of other retroviral pol genes.

The substantial elimination of RNase H activity of the pol gene product may be effected by deletion of at least a part of the pol gene encoding RNase H.

In a further aspect of the invention, there is provided modified retroviral genomes of the invention

including a segment encoding at least one antigenic marker.

In one specific illustrative embodiment of this aspect of the invention, the sequence encoding the at least one antigenic marker is inserted into the gag gene at an antigenically active insertion site and specifically at the PstI site at nucleotide 1415 of the gag gene of HIV-1 LAI isolate or the corresponding location of other retroviral gag genes. One specific segment comprises from 1 to 4 copies of a DNA sequence selected from the group consisting of:

- (a) 5' GCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAAT 3';  
(SEQ ID NO: 5);
- (b) 3' CGTAAGCTGTGATCTTTATCTTATTATCTTCAACTTTTA 5';  
(SEQ ID NO: 6); and
- (c) DNA sequences that hybridize with (a) or (b) under stringent conditions, particularly sequences that have at least about 90% sequence identity with the sequence of (a) or (b).

A variety of hybridization conditions may be employed to achieve varying degrees of selectivity of hybridization. For a high degree of selectivity, stringent conditions are used to form duplexes, such as low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions may be required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex.

In a yet further embodiment of the present invention, there is provided a nucleic acid molecule encoding a non-infectious retrovirus-like particle of the invention, comprising a modified retroviral genome

deficient in long terminal repeats and containing gag,  
pol and env genes in their natural genomic arrangement  
with the env gene being modified to provide therein a  
segment encoding an antigenic anchor sequence to anchor  
5 the env gene product to the retrovirus-like particle,  
whereby the modified env gene encodes a modified env gene  
product in which endogenous anchoring function of env has  
been replaced by the antigenic anchor sequence.

In one specific illustrative embodiment of this  
10 aspect of the invention, the segment encoding the  
antigenic marker sequence is inserted into the env gene,  
specifically between nucleotide 7777 and 7778 of the env  
gene of the HIV-1 LAI isolate or the corresponding  
location of other retroviral env genes. One specific  
15 segment encoding the anchor sequence includes a DNA  
sequence selected from the group consisting of:

- (a) 5' TGGATCCTGTGGATTCCTTTGCCATATCATGCTTTTGTCTTG  
TGTGTTTTGCTGGGGTTCATCATGTGG 3'; (SEQ ID NO: 7);
- (b) 3' ACCTAGGACACCTAAAGGAAACGGTATAGTACGAAAAACGAAAC  
20 ACAACAAAACGACCCCAAGTAGTACACC 5'; (SEQ ID NO: 8); and
- (c) DNA sequences that hybridize with (a) or (b)  
under stringent conditions, particularly sequences that  
have at least about 90% sequence identity with the  
sequences of (a) or (b).

25 Another specific segment encoding the anchor  
sequence includes a DNA sequence selected from the group  
consisting of:

- (a) 5' TCAACAGTGGCAAGTTCCTAGCACTGGCAATCATGATAGC  
TGGTCTATCTTTTGGATGTGTTCCAATGGGTCATTGCAG 3'; (SEQ ID  
30 NO: 9)
- (b) 3' AGTTGTCACCGTTCAAGGGATCGTGACCGTTAGTACTATCGA  
CCAGATAGAAAAACCTACACAAGGTTACCCAGTAACGTC 5'; and (SEQ  
ID NO: 10); and
- (c) DNA sequences that hybridize with (a) or (b)  
35 under stringent conditions, particularly sequences that  
have at least about 90% sequence identity with the

sequences of (a) or (b). Another specific segment encoding the anchor sequence is selected from the group consisting of:

5 (a) 5' TGGATCCTGTGGATTTCCTTTGCCATATCATGCTTTTGGCTT  
TGTGTTGTTTGCTGGGGTTCATCATGTGGGCCTGCCAAAAGGCAACATT  
AGGTGCAACATTTGCATTTGATAGTAAAGAAGAGTGGTGCAGAGAGAAAA  
AAGAGCAGTGGGAA 3'; (SEQ ID NO: 11);

(b) 3' ACCTAGGACACCTAAAGGAAACGGTATAGTACGAAAAACGAA  
ACACAACAAACGACCCCAAGTAGTACACCCGGACGGTTTTTCCGTTGTAA  
10 TCCACGTTGTAAACGTAAACTATCATTTCTTCTCACCACGTCTCTCTTTT  
TTCTCGTCACCCTT 5'; and (SEQ ID NO: 12); and

(c) DNA sequences that hybridize with (a) or (b) under stringent conditions, particularly sequences that have at least about 90% sequence identity with the  
15 sequence of (a) or (b).

The present invention further includes, in an additional aspect, an immunogenic composition capable of eliciting a retroviral specific immune response, comprising the retrovirus-like particles or nucleic acid  
20 molecule provided herein, and a carrier therefor. Such composition may be formulated for mucosal or parenteral administration, by oral, anal, vaginal or intranasal routes. The immunogenic composition may comprise at least one other immunogenic or immunostimulating  
25 material, specifically an adjuvant, such as aluminum phosphate, aluminum hydroxide, QS21, Quil A, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octadecyl ester of an amino acid, a muramyl dipeptide, a lipoprotein or Freund's incomplete adjuvant.

30 In a further aspect, the present invention includes a method of immunizing a host to produce a retroviral specific immune response, comprising administering to the host an immunoeffective amount of the immunogenic composition provided herein.

35 The present invention also includes diagnostic procedures and kits utilizing those materials.

Specifically, in another aspect of the invention, there is provided a method of determining the presence of antibodies specifically reacting with retroviral antigens in a sample, comprising the steps of (a) contacting the sample with the non-infectious retrovirus-like particle provided herein to produce complexes comprising the non-infectious retrovirus-like particles and any said antibodies present in the sample specifically reactive therewith; and (b) determining production of the complexes.

In an additional aspect of the invention, there is provided a method of determining the presence of retroviral antigens in a sample, comprising the steps of (a) immunizing a host with the immunogenic composition provided herein to produce retroviral antigen-specific antibodies; (b) contacting the sample with the retroviral antigen-specific antibodies to produce complexes comprising any retrovirus antigens in the sample and retroviral antigen-specific antibodies; and (c) determining production of the complexes.

A further aspect of the invention provides a diagnostic kit for detecting the presence of retroviral antigens in a sample comprising (a) at least one such retroviral antigen-specific antibody provided herein; (b) means for contacting the at least one antibody with the sample to produce a complex comprising any retroviral antigens in the sample and the retroviral antigen-specific antibodies; and (c) means for determining production of the complex.

Advantages of the present invention include:

- an immunogenic retrovirus-like particle comprising gag, pol and env gene products in their natural conformations rendered non-infectious and non-replicating by a plurality of mutations; and
- an immunogenic retrovirus-like particle immunologically distinguishable from a virulent retrovirus.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a construction scheme of a plasmid (pMTHIV-A) encoding a retrovirus-like particle having a modification to the gag gene product in accordance with one embodiment of the invention;

Figure 2 shows a construction scheme of a plasmid (pMTHIVBRU) encoding a retrovirus-like particle having a modification to both the gag and pol gene products in accordance with a further embodiment of the invention;

Figure 3 shows a construction scheme of a plasmid (p83-19) encoding a retrovirus-like particle having a modification in the env gene product in accordance with a further embodiment of the invention;

Figure 4 shows a construction scheme of plasmid pSeBS-HA2 containing a heterologous anchor sequence in the env gene;

Figure 5 shows a flow diagram for gene assembly-aided mutagenesis;

Figure 6 shows a construction scheme of a plasmid (pMTHIVHA2-701) encoding a retrovirus-like particle containing an antigenic marker comprising a portion of the transmembrane component of human influenza hemagglutinin glycoprotein;

Figure 7 shows a construction scheme of a plasmid (pMTHIVmHA2) encoding a retrovirus-like particle containing a non-naturally occurring marker;

Figure 8 shows a construction scheme for a plasmid (pMTHIVMNmHA2-5) encoding a retrovirus-like particle containing a non-naturally occurring marker;

Figure 9 shows details of an oligonucleotide encoding an antigenic epitope from tobacco mosaic virus inserted into the gag gene product of a non-infectious non-replicating retrovirus-like particle;

Figure 10 shows a construction scheme of plasmids encoding retrovirus-like particles having antigenic epitopes from tobacco mosaic virus;



Figure 11 shows an immunoblot analysis of antigenically marked retrovirus-like particles (pseudovirions);

5 Figure 12 shows an immunoblot analysis of antigenically marked retrovirus-like particles to demonstrate inclusion of the antigenic marker in the gag gene product;

10 Figure 13 shows the immune response in guinea pigs immunized with retrovirus-like particles, antigenically-marked by inclusion of the mHA2 sequence;

Figure 14 shows the immune response in guinea pigs immunized with retrovirus-like particles antigenically-marked by inclusion of the TMV marker sequence;

15 Figure 15 shows the location of an immunodominant region in the envelope glycoprotein gp120 of HIV-1 for modification to provide a non-infectious retrovirus-like particle antigenically distinguishable from HIV-1; and

20 Figure 16 shows an immunoblot analysis of retrovirus-like particles containing envelope glycoprotein gp120 of a clinical, primary isolate of HIV-1.

#### GENERAL DESCRIPTION OF INVENTION

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention  
25 have many applications in the fields of vaccination, diagnosis, treatment of HIV infections, and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

30 Referring to Figures 1 and 2, there is illustrated the construction of a vector pMTHIVBRU (ATCC designation 75852) containing a modified retroviral genome deficient in long terminal repeats, primer binding site and an RNA packaging sequence, and containing gag, pol and env genes  
35 in their natural genomic arrangement. The pol gene of pMTHIVBRU has been modified by deletion of a portion

thereof to substantially remove the reverse transcriptase and integrase activities thereof. Furthermore, in this particular illustrated embodiment of the invention, an oligonucleotide has been inserted within the deleted pol gene to introduce three stop codons in three different reading frames to prevent remaining sequences of integrase from being translated. The gag gene of pMTHIVBRU has also been modified to replace the two cysteine residues (Cys<sup>392</sup> and Cys<sup>395</sup>) in the first Cys-His box by serines.

Thus, plasmid pMTHIVBRU encodes an HIV-like particle deficient in a plurality of elements required for infectivity and/or replication of HIV but dispensable for virus-like particle production.

Plasmid pMTHIVBRU encodes an HIV-like particle with an envelope protein corresponding to that of the HIV-1<sub>LAI</sub> isolate. Referring to Figure 3, there is shown a plasmid p83-19 in which the LAI envelope of pMTHIVBRU has been substantially replaced by the MN envelope sequence. Thus, plasmid p83-19 encodes an HIV-like particle deficient in a plurality of elements required for infectivity and/or replication of HIV but dispensable for virus-like particle production, and contains as the env gene product substantially the envelope of HIV-1 isolate MN. The HIV-like particle may contain other env gene products, particularly from clinical isolates from HIV-1 infected patients, such as a primary HIV-1 isolate from clades A, B, C, D, E and O, including the specific isolate bx08. The env gene products also may be a chimera of the gp120 protein from one source and the remainder from another source, such as MN/LAI, bx08/LAI and clades/LAI chimeras (Figure 15).

Referring to Figures 4 to 6, there is illustrated the construction of a vector pMTHIVHA2-701 containing a modified HIV genome deficient in long terminal repeats, primer binding site and an RNA packaging sequence, and

containing gag, pol and env genes in their natural genomic arrangement. The env gene in pMTHIVHA2-701 has been modified to provide therein a gene encoding a different anchor sequence to anchor the env gene product to the retrovirus-like product, whereby the modified env gene encodes a modified env gene product in which endogenous anchoring function of env has been replaced by the different anchor sequence. In retrovirus-like particles encoded by pMTHIVHA2-701 an immunodominant epitope of gp41 (which provides endogenous anchoring function) is no longer expressed. Thus, such retrovirus-like particles are antigenically marked in a negative manner by the absence of an amino acid sequence corresponding to an epitope of a retroviral protein. The different anchor sequence may itself be antigenic to further provide a positive non-retroviral or non-HIV retroviral antigenic marker for the retrovirus-like particles.

In this particular illustrated embodiment of the invention, a 135-bp sequence comprising a coding DNA fragment and a stop codon from the human influenza virus HA2 gene was inserted between nucleotides 7777 (G) and 7778 (A) of the HIV-1<sub>LAI</sub> envelope gene to prevent synthesis of the HIV-1<sub>LAI</sub> gp41 transmembrane glycoprotein. Plasmid pMTHIVHA2-701 thus encodes an HIV-like particle wherein the gp41 transmembrane glycoprotein anchoring function has been replaced by an anchor sequence from the human influenza virus HA2 protein and the HA2 protein further provides an antigenic marker.

Referring to Figure 7, there is illustrated plasmid pMTHIVmHA2 which is similar to pMTHIVHA2-701 but contains as the antigenic marker sequence replacing the endogenous anchoring function of env, an amino acid sequence with no homology to known naturally occurring proteins.

Referring to Figure 8, there is illustrated a vector pMTHIVMNmHA2-5 (ATCC designation 75853) containing a

modified HIV genome deficient in long terminal repeats, primer binding site and an RNA packaging sequence and containing gag, pol and env genes in their natural genomic arrangement. The pol gene of pMTHIVMnMHA2-5 has  
5 been modified by deletion of a portion thereof to substantially remove the reverse transcriptase and integrase activities thereof. Furthermore, an oligonucleotide was inserted within the deleted pol gene to introduce three stop codons in three different reading  
10 frames to prevent remaining sequences of integrase from being translated. The gag gene of pMTHIVMnMHA2-5 has also been modified to replace the two cysteine residues in the first Cys-His box of gag by serines. In pMTHIVMnMHA2-5, the endogenous anchoring function of env  
15 has been replaced by an amino acid sequence with no known homology to naturally occurring proteins. HIV-like particles produced from Vero cells (and other vaccine-quality cell lines, including MCR5 cells, primary monkey kidney (African Green) cells, WI38 cells and baby hamster  
20 kidney cells) transfected with plasmid pMTHIVMnMHA2-5 were purified and used to immunize guinea pigs. Antisera were collected and assayed by ELISA for anti-V3 (i.e. anti-envelope) antibodies and anti-mHA2 (i.e. anti-antigenic marker) antibodies as shown in Table 1. These  
25 experiments were repeated in other guinea pigs and extended to the assay for anti-gag antibodies and the results obtained are shown in Figure 13. These results indicate that the gag and env gene products are present in substantially its native conformation and that the  
30 antigenic marker is immunogenic.

Although particular retrovirus-like particles have been described in which endogenous anchoring function of env has been replaced by the antigenic anchor sequence of particular natural and unnatural proteins, it is  
35 appreciated that many variations, adaptations and modifications can be made to the particular means by

which the endogenous anchoring function can be replaced without departing from the essence of the invention.

Referring to Figures 9 and 10, there is illustrated plasmids (pHIV-T1; pHIV-T2 (ATCC designation 75,851);  
5 pHIV-T3 and pHIV-T4) containing between one and four copies of a DNA sequence encoding an antigenic epitope from TMV. In the particular embodiments shown, the TMV epitope is inserted into the gag gene of HIV to produce a hybrid gag gene product, and the plasmids are deficient  
10 in the plurality of elements required for infectivity and/or replication of HIV but dispensable for virus-like particle production as described above. Stable cell lines were produced using plasmids pHIV-T1, pHIV-T2 (ATCC designation 75852), pHIV-T3 and pHIV-T4 (containing 1, 2,  
15 3 and 4 copies of the antigen epitope, respectively) that produced HIV-like particles containing the antigenic marker inserted into the gag protein. These HIV-like particles were purified and their reactivity with anti-HIV monoclonal antibodies (Figure 11) and anti-TMV marker  
20 antiserum (Figure 12) determined. The results are shown in Figures 11 and 12 and indicate that the HIV-like particles contain gp120, gp41 and p24 in substantially their natural conformations and that the TMV marker is able to be recognized by anti-marker antibodies.

25 Purified HIV-like particles produced by plasmid pHIV-T2 were used to immunize guinea pigs. Antisera were collected and assayed by ELISA for anti-gag antibodies anti-V3 (i.e. anti-envelope) antibodies and anti-TMV (i.e. anti-antigenic marker) antibodies as shown in  
30 Figure 14. These results indicate that the gag and env gene products are present in substantially their native conformation and that the antigenic marker is immunogenic.

Further, a viral infection inhibition assay was  
35 carried out on the guinea pig sera from these immunizations. Neutralizing antibodies were formed, as

shown in Table 2. The generation of such neutralizing antibodies indicates the potential utility of the retrovirus-like particles as vaccine candidates and in diagnostic applications.

5 Referring to Figure 16, there is shown an immunoblot analysis of retrovirus-like particles (pseudovirion) expressing envelope glycoproteins from the clinical (primary) isolate bx08. HIV-1 bx08 is a clinical isolate from clade B isolated in France. This analysis  
10 demonstrates the expression of the envelope glycoproteins from clinical isolates in a molecule containing non-clinical isolate gag protein.

While specific embodiments of the marker sequences, which may also be an anchor sequence, are described  
15 herein, it is apparent that any other convenient amino acid sequence providing marker and/or anchoring function may be employed herein, including the absence of an amino acid sequence that corresponds to an epitope of a retroviral protein. The absence of an amino acid  
20 sequence corresponding to an epitope of retroviral protein may involve mutagenesis of one or more amino acids from an immunodominant region of the env gene product, as shown in Table 3, to substantially prevent recognition of the immunodominant region in the resulting  
25 mutation by sera from retroviral-infected hosts. The amino acid sequence providing marker function may comprise a non-naturally occurring antigenic sequence which has no homology to known protein. An example of such sequence is the mutant HA2 sequence described above.  
30 Other examples may include antigenic regions of non-human or non-mammalian protein, such as non-human or non-mammalia pathogenic or comensual organisms. An example of such sequence is the TMV described above.

It is clearly apparent to one skilled in the art,  
35 that the various embodiments of the present invention have many applications in the fields of vaccination,

diagnosis, treatment of HIV infections, and the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

5 Vaccine Preparation and Use

It has been shown that an immunogenic preparation in accordance with the invention can elicit an immune response. One possible use of the present invention is, therefore, as the basis of a potential vaccine against  
10 retroviral diseases including AIDS and AIDS-related conditions. In a further aspect, the invention thus provides a vaccine against AIDS and AIDS-related conditions, comprising an immunogenic composition in accordance with the invention.

15 Immunogenic compositions, suitable to be used as vaccines, may be prepared from non-infectious retrovirus-like particles as disclosed herein. The immunogenic composition elicits an immune response which produces antibodies that are antiviral. Should the vaccinated  
20 subject be challenged by a retrovirus, such as HIV, the antibodies bind to the virus and thereby inactivate it.

Vaccines may be prepared as injectables, as liquid solutions or emulsions. The non-infectious retrovirus-like particles may be mixed with pharmaceutically-  
25 acceptable excipients which are compatible with the retrovirus-like particles. Excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The vaccine may further contain auxiliary substances, such as wetting or emulsifying agents, pH  
30 buffering agents, or adjuvants to enhance the effectiveness of the vaccines. Methods of achieving an adjuvant effect for the vaccine include the use of agents, such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in  
35 phosphate buffered saline and other adjuvants, including QS21 and incomplete Freund's adjuvant. Vaccines may be

administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed incipients, such as pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations or powders and contain 10 to 95% of the retrovirus-like particles of the invention.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as is therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the retrovirus-like particles. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. One example of an immunization schedule is at least one pre-immunization with a retrovirus-like particle, according to the present invention followed by at least one secondary immunization



with a synthetic peptide described in published European Patent Publication Number 0 570 980, assigned to the assignee hereof. The dosage of the vaccine may also depend on the route of administration and will also vary according to the size of the host.

Nucleic acid molecules encoding the retrovirus-like particles of the present invention may also be used directly for immunization by administration of the nucleic acid molecules directly, for example by injection to a host. Processes for the direct injection of DNA into test subjects for genetic immunization are described in, for example, Ulmer et al, 1993 (a list of references appears at the end of the disclosure and each of the listed references is incorporated by reference without further reference thereto).

Molecules in accordance with the invention may further find use in the treatment (prophylactic or curative) of AIDS and related conditions, by acting either to displace the binding of the HIV virus to human or animal cells or by disturbing the 3-dimensional organization of the virus.

A further aspect of the invention thus provides a method for the prophylaxis or treatment of AIDS or related conditions, comprising administering an effective amount of an immunogenic composition in accordance with the invention.

#### Immunoassays

The retrovirus-like particles of the present invention are useful as immunogens, as antigens in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays, or procedures known in the art for the detection of anti-retroviral (for example, HIV) HIV antibodies and retroviral antigen (for example, HIV). In ELISA assays, the retrovirus-like particles are immobilized onto a selected surface, for example a

surface capable of binding proteins, such as the wells of a polystyrene microtitre plate. After washing to remove incompletely adsorbed retrovirus-like particles, a non-specific protein, such as a solution of bovine serum albumin (BSA) or casein, that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of non-specific adsorption sites on the immobilizing surface and thus decreases the background caused by non-specific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials to be tested, in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures such as of the order of about 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween, or a borate buffer.

Following formation of specific immunocomplexes between the test sample and the bound retrovirus-like particles, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity that will generate, for example, a colour development upon incubating with an appropriate

chromogenic substrate. Quantification may then be achieved by measuring the degree of colour generation using, for example, a visible spectra spectrophotometer.

In one diagnostic embodiment where it is desirable to identify antibodies that recognize a plurality of HIV isolates, a plurality of immunologically distinct retrovirus-like particles of the present invention are immobilized onto the selected surface. Alternatively, when the anti-HIV antibodies recognize epitopes that are highly conserved among various HIV isolates (for example, a B-cell epitope from gag or gp41) a single or a limited number of retrovirus-like particles may be immobilized. In a further diagnostic embodiment where it is desirable to specifically identify antibodies that recognize a single HIV isolate (for example, LAI, MN, SF2 or HXB2) a single particular retrovirus-like particle of the present invention may be immobilized. This further diagnostic embodiment has particular utility in the fields of medicine, clinical trials, law and forensic science where it may be critical to determine the particular HIV isolate that was responsible for the generation of an immune response including an antibody response.

In a further diagnostic embodiment, it may be desirable to specifically identify immunologically distinct retroviruses, for example, HIV isolates that belong to different clades. Immunologically distinct HIV isolates may include for example, LAI, MN, SF2, HXB2 or a primary HIV-1 isolate. In this diagnostic embodiment, a particular retrovirus-like particle of the present invention is useful for generating antibodies including monoclonal antibodies that specifically recognize such an immunologically distinct HIV isolate.

It is understood that a mixture of immunologically distinct retrovirus-like particles may be used either as an immunogen in, for example, a vaccine or as a diagnostic agent. There may be circumstances where a

mixture of retrovirus-like particles are used to provide cross-isolate protection and/or diagnosis. In this instance, the mixture of immunogens is commonly referred to as a "cocktail" preparation.

5       The present invention advantageously provides retrovirus-like particles comprising gag, pol and env gene products substantially in their natural conformations. Such retrovirus particles will thus be recognized by conformational anti-HIV antibodies (such as  
10 anti-env antibodies) that may not recognize the HIV antigen in a denatured form or a synthetic peptide corresponding to such an HIV antigen. The retrovirus-like particles of the invention are therefore particularly useful as antigens and as immunogens in the  
15 generation of anti-retroviral antibodies (including monoclonal antibodies) in diagnostic embodiments.

In addition, the presence of the marker generates a specific immune response thereto the detection of which by the methods described above enables the ready  
20 distinction between immunization of a host with the immunogenic compositions provided herein compared to material infection by a virulent retrovirus. The ability to effect such diagnosis and differentiation has advantageous utility in the fields of epidemiology,  
25 clinical trials, forensic science and immunology.

#### Other Uses

Molecules which bind to the retrovirus-like particles on which the invention is based, particularly antibodies, antibody-related molecules and structural  
30 analogs thereof, are also of possible use as agents in the treatment and diagnosis of AIDS and related conditions.

Variants of antibodies (including variants of antigen binding site), such as chimeric antibodies,  
35 humanized antibodies, veneered antibodies, and engineered antibodies that are specific for the retrovirus-like

particles of the invention are included within the scope of the invention.

Antibodies and other molecules which bind to the retrovirus-like particles of the present invention can be  
5 used for therapeutic (prophylactic and curative) and diagnostic purposes in a number of different ways, including the following:

For passive immunization by suitable administration of antibodies, possibly humanized antibodies, to HIV  
10 infected patients.

To activate, complement or mediate antibody dependent cellular cytotoxicity (ADCC) by use of antibodies of suitable subclass or isotype (possibly obtained by appropriate antibody engineering) to be  
15 capable of performing the desired function.

For targeted delivery of toxins or other agents, for example, by use of immunotoxins comprising conjugates of antibody and a cytotoxic moiety, for binding directly or indirectly to cell-surface exposed HIV proteins of HIV-  
20 infected cells (for example, gp120).

For targeted delivery of highly immunogenic materials to the surface of HIV-infected cells, leading to possible ablation of such cells by either the humoral or cellular immune system of the host.

25 For detection of HIV, using a variety of immunoassay techniques.

Thus, in yet a further diagnostic embodiment, the immunogenic compositions of the present invention (individually, or as mixtures including cocktail  
30 preparations) are useful for the generation of HIV antigen specific antibodies (including monoclonal antibodies) that can be used to detect HIV or antigens, or neutralize HIV in samples including biological samples.

35 In an alternative diagnostic embodiment, the retrovirus-like particles of the present invention can be

used to specifically stimulate HIV specific T-cells in biological samples from, for example, HIV-infected individuals for diagnosis or therapy.

#### Biological Deposits

5 Certain plasmids that encode retrovirus-like particles according to aspects of the present invention that are described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at 12301 Parklawn Drive, Rockville,  
10 Maryland, USA, 20852, pursuant to the Budapest Treaty and prior to the filing of this application. Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application. The invention described and claimed  
15 herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent retrovirus-like particles as described in this  
20 application are within the scope of the invention.

#### Deposit Summary

<u>Plasmid</u>	<u>ATCC Designation</u>	<u>Date Deposited</u>
pMTHIVBRU	75,852	August 4, 1994
pMTHIVMNmHA2-5	75,853	August 4, 1994
25 pHIV-T2	75,851	August 4, 1994

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of  
30 illustration and are not intended to limit the scope of the invention. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations. Immunological and recombinant DNA methods may not be explicitly  
35 described in this disclosure but are well within the scope of those skilled in the art.

**EXAMPLES**

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these EXAMPLES are amply reported in the scientific literature and are well within the ability of those skilled in the art.

**Example 1:**

This Example describes the construction of plasmid pMTHIVBRU.

Plasmid pMTHIVBRU was constructed as shown in Figures 1 and 2. This plasmid is a modification of the expression vector pMTHIVd25 described in Rovinski et al 1992 (the literature references are identified at the end of the specification) and which contains an RNA packaging deletion, and was engineered to contain a series of mutations/deletions. Thus, a Cys-His box mutation included replacements of two cysteine codons (in SEQ ID NO: 13) with two serine codons in the first Cys-His box (SEQ ID NO: 14) of the gag protein as shown in Figure 1. This was accomplished by a PCR-based mutagenesis method. Two primers were synthesized: the upstream primer having the sequence 5'-GGACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAG-3' (SEQ ID NO: 15), comprising nucleotides 1,507 to 1,567 of HIV-1<sub>LAI</sub>, (all nucleotide numbering is according to Wain-Hobson et al., 1985) with a SpeI site at the 5'-end; and the downstream primer having the sequence 5'-CTCGGGCCCTGCAATTTCTGGCTATGTGCCCTTC TTGCCACTATTGAAACTCTTAACAATC-3' (SEQ ID NO: 16), being the reverse complement of nucleotides 2,011 to 1,953 with an ApaI site at the 5'-end. In the downstream primer, two adenosine residues representing the reverse complement of nucleotides 1,963 and 1,972 (Wain Hobson et al, 1985; Myers et al, 1990) were changed to thymidine, resulting in the replacement of the two cysteines at amino acid positions 392 and 395 of the gag gene product with two serines (Figure 1). These two primers were used

to amplify the SpeI-ApaI DNA fragment (nucleotides 1507 to 2006) of pMTHIV (Rovinski et al, 1992) which was used as a template. The PCR-amplified SpeI-ApaI fragment was purified by agarose gel electrophoresis and digested with  
5 restriction enzymes SpeI and ApaI. This fragment was used to replace the corresponding fragment in pMTHIVd25 (Rovinski et al, 1992). The resulting plasmid was named pMTHIV-A, which contains both the RNA packaging sequence deletion and the Cys-His box mutation.

10 In order to delete the reverse transcriptase and integrase, two BalI recognition sites at nucleotides 2,655 and 4,587 of HIV-1<sub>LAI</sub>, were used (Figure 2). The 1.9-kbp fragment between the two BalI sites contains DNA sequences encoding more than 95% of the reverse  
15 transcriptase and the first 108 amino acids of the integrase. The plasmid pMTHIV-A was digested with BalI. After removing the 1.9-kbp BalI fragment by gel electrophoresis, the remaining portion of the plasmid was ligated with a double-stranded oligonucleotide: 5'-  
20 GTATAAGTGAGTAGCGGCCGCAC-3' (only one strand is shown - SEQ ID NO: 17) which contains three stop codons in three different reading frames to prevent the remaining sequences of integrase from being translated. The resulting plasmid was termed pMTHIVBRU.

25 Example 2:

This Example describes the construction of plasmids encoding HIV-like particles containing antigenically marked envelope anchors.

Plasmid p83-19 was constructed from expression  
30 vector pMTHIVBRU, as shown in Figure 3. This plasmid contains a hybrid envelope gene which was engineered by replacing DNA encoding most of gp120<sub>LA1</sub>, with the cognate DNA encoding gp120<sub>MN</sub>. This was accomplished by replacing a KpnI/BglII DNA fragment (nucleotides 6379 to 7668) from  
35 HIV-1<sub>LA1</sub>, with a KpnI/BglII DNA fragment (nucleotides 6358 to 7641) from HIV-1<sub>MN</sub>.



Plasmid pMTHIVHA2-701 was constructed from expression vectors pBT1 (Alizon et al, 1984) and pMTHIVd25 (Rovinski et al, 1992), as shown in Figures 4 to 6. The pMTHIVHA2-701 vector contains a 135-bp sequence comprising a coding DNA fragment and a stop codon from the human influenza virus HA2 gene (Min Jou et al, 1980), inserted between nucleotides 7777(G) and 7778(A) of the HIV-1<sub>LAI</sub> envelope gene (Wain-Hobson et al, 1985; Myers et al, 1990). The stop codon was inserted to prevent synthesis of the HIV-1<sub>LAI</sub> gp41 transmembrane glycoprotein. A SalI (nucleotide 5821)/BamHI (nucleotide 8522) DNA fragment from pBT1 was subcloned into pSelect (Promega) to produce pSeBS (Figure 4). The latter plasmid was used for insertion of the 135-bp by a procedure termed herein as 'gene assembly-aided mutagenesis (GAAM)'. A mutagenic primer, which was designed to contain the 135-bp sequence comprising a coding DNA fragment from the human influenza virus HA2 gene (Min Jou et al, 1980), was assembled as shown in Figure 5. Oligonucleotide I is a 99mer containing (from 3' to 5') 30 bases complementary to nucleotides 7748 to 7777 of HIV-1<sub>LAI</sub> (Wain-Hobson et al, 1985; Myers et al, 1990) and 69 bases which are complementary to HA2 gene sequences (Min Jou et al, 1980) encoding amino acids 180 to 202 of the HA2 protein. Oligonucleotide II is a 96mer comprising (from 3' to 5') i) 60 bases complementary to HA2 gene sequences which encode amino acids 203 to 221 of the HA2 protein and contain the HA2 stop codon (Min Jou et al, 1980), ii) 6 bases (ATCATT - SEQ ID NO: 18) defining two more stop codons, and iii) 30 bases complementary to nucleotides 7778 to 7807 of HIV-1<sub>LAI</sub>, (Wain-Hobson et al, 1985; Myers et al, 1990). Oligonucleotide III is a bridging 30mer having 15 nucleotides complementary to the 5'-end of oligonucleotide I and 15 nucleotides complementary to the 3'-end of oligonucleotide II. Ten picomoles of

oligonucleotides I and II were mixed with 20 picomoles of oligonucleotide III and phosphorylated at 37°C for 1.5 h in 20 µl kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM DTT, and 0.5 mM ATP) containing 2  
5 units of T4 polynucleotide kinase. The oligonucleotides were annealed by heating the mixture to 95°C for 5 min and subsequently cooling it slowly to room temperature. To this mixture was added 3 µl of 10 x ligase buffer (0.5 M Tris-HCl, pH 7.4, 0-1 M MgCl<sub>2</sub>, 0.1 M DTT, 10 mM  
10 Spermidine, and 1 mg/ml BSA), 3 µl of 10 mM ATP, and 5 units of T4 DNA ligase, and the ligation mixture was incubated overnight at 16°C to complete the assembly of the mutagenic primer (Figure 5). This primer was used in the mutagenesis procedure without further purification.

15 Mutagenesis was performed using the Altered Sites in vitro Mutagenesis System from Promega (Madison, WI). The template for mutagenesis consisted of the pSeBS plasmid (Figure 4) which contained the 2.7-kbp SalI/BamHI DNA fragment of the HIV-1<sub>LA1</sub> envelope gene (nucleotides 5821  
20 to 8522) cloned into the pSelect phagemid vector provided in the mutagenesis kit. Following the mutagenesis procedure, putative clones were identified by colony hybridization with a <sup>32</sup>P-labelled oligonucleotide III probe. Positive clones were confirmed by DNA sequencing.  
25 One of these clones, designated pSeBS-HA2, was used for the construction of the final vector. To this end, the modified SalI/BamHI insert from pSeBS-HA2 was subcloned into pMTHIVd25-dSalI; the latter is a plasmid derived from pMTHIVd25 (Rovinski et al, 1992) by partial  
30 digestion with SalI followed by Klenow treatment to eliminate the SalI site within the plasmid backbone. The final expression construct was designated pMTHIVHA2-701.

An expression vector, pMTHIVmHA2 (shown in Figure 7) containing a heterologous DNA sequence inserted between  
35 nucleotides 7777 (G) and 7778 (A) of the HIV-1<sub>LA1</sub> envelope gene (Rovinski et al, 1992; Wain-Hobson et al, 1985) was

engineered as described above. In this case, a 134-bp sequence, comprising a coding DNA fragment from the human influenza virus HA2 gene (Min Jou et al, 1990) and 68 nucleotides that, when fused to the HA2 sequences, encodes an amino acid sequence with no homology to known naturally occurring proteins, was inserted downstream of nucleotide 7777 of HIV-1<sub>LAI</sub> (Figure 7). The insertion resulted in a frameshift in the translation of HIV-1<sub>LAI</sub> coding sequences, and the creation of a stop codon (TAG) to prevent synthesis of the gp41 transmembrane glycoprotein of HIV-1<sub>LAI</sub>. The final expression construct was designated pMTHIVmHA2 (Figure 7).

Plasmid pMTHIVMNmHA2-5 was constructed from expression vectors p83-19 and pMTHIVmHA2 as shown in Figure 8. This plasmid was designed to have all of the mutations of elements required for infectivity and/or replication of p83-19 and to contain the 134-bp insert sequence of pMTHIVmHA2 (Figure 7). To this end, p83-19 was digested with BglII (nucleotide 7,641) and XhoI (nucleotide 8,944) to remove a 1276-bp DNA fragment which was replaced by the cognate BglII/XhoI fragment of pMTHIVmHA2.

#### Example 3:

This Example describes the construction of plasmids encoding HIV-like particles containing antigenic epitopes from TMV.

Plasmids pHIV-T1, pHIV-T2, pHIV-T3, and pHIV-T4 represent modified versions of the p83-19 construct in that they contain, respectively, either one, two, three, or four copies of a double-stranded oligonucleotide (Figures 9, 10 and 11) comprising at least one antigenic epitope (Westhof et al, 1984; Trifilleff et al, 1991) from TMV coat protein. The construction of these four vectors is illustrated in Figures 9 and 10. To engineer all constructs, plasmid pMTHIV-A (Figure 1) was first digested with SacII and ApaI to isolate a 1,328-bp DNA

fragment which was then subcloned into pBluescript (Stratagene) . The recombinant plasmid was then digested with PstI which cleaves HIV-1<sub>LAI</sub> DNA at nucleotide 1,415 within the gag gene. Subsequently, either one, two, 5 three, or four copies of the double-stranded oligonucleotide shown in Figure 9 (coding strand: SEQ ID NO: 19, complementary strand: SEQ ID NO: 20, encoded amino acids: SEQ ID NO: 21) were inserted into this restriction site. Finally, the resulting recombinant 10 plasmids were digested with SacII and ApaI to release the modified insert which was then cloned into the cognate region of plasmid p83-19 (Figure 10).

The expression of retrovirus-like particles containing either the MHA2 epitope or various copies of 15 the TMV epitope is depicted in Figure 11. Vero cells were grown to 80% confluency and transfected with 20 µg of plasmid DNA by the transfinity (BRL) calcium phosphate procedure. Culture supernatants were analyzed for protein expression at 48 h post-transfection. Culture 20 media (10 ml) from cells transfected with individual expression constructs were collected and clarified by centrifugation at 2,000 x g (Sorvall RT 6000B; Dupont Company, Wilmington, Del.) for 15 min at 4°C. Retrovirus-like particles were isolated by ultra- 25 centrifugation. Pelleted particles were suspended to 40 µl of TNE, mixed with 10 µl of 5x Laemmli sample buffer and boiled for 3 min. Viral proteins were then separated by SDS PAGE and transferred to Immobilon membranes (Millipore, Bedford, Mass.). Membranes were blocked with 30 BLOTTO buffer (PBS containing 5% Carnation instant nonfat dry milk, 0.0001% wt/vol thimerosal, and 0.01% vol/vol antifoam A emulsion) for 2 h at 25°C and then incubated with appropriate dilutions of antibodies overnight at 4°C. Filters were then incubated with a goat anti-mouse 35 immunoglobulin G antibody conjugated to alkaline phosphatase (Promega, Madison, Wis.) and reacted with the

alkaline phosphatase chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate  $\rho$ -toluidine salt (BRL). A cocktail of anti-gp120, anti-gp41, and anti-p24 antibodies was used in Panel A. A mixture of anti-gp120 and anti-p24 antibodies was used in Panel B.

The results shown in Figure 11 demonstrate that the antigenically marked HIV-like particles produce gp120, gp41 and p24 substantially in their natural conformations.

Example 4:

This Example describes the immunogenicity and immunoreactivity of antigenically marked HIV-like particles.

One of plasmids pHIV-T1, pHIV-T2, pHIV-T3, or pHIV-T4 (Figure 10) was co-transfected with plasmid pSV2neo into Vero cells, and stable cell lines were established that produce HIV-like particles. HIV-like particles were purified, and their reactivity to immune sera from guinea pigs immunized with a peptide corresponding to the TMV marker inserted into the gag gene product was determined by immuno blot analysis. To obtain the immune sera, guinea pigs were immunized with 100  $\mu$ g of a peptide consisting of the TMV marker conjugated to KLH and adjuvanted in Freund's complete adjuvant. All animals were boosted three times at 3-week intervals with the same peptide adjuvanted in Freund's incomplete adjuvant. Immune sera were collected two weeks after the last booster shots. The results, presented in Figure 12, illustrate the reactivity of the immune sera to various forms of the gag gene product present in the various HIV-like particles and demonstrate the antigenicity of the TMV marker in the context of a modified HIV-1-like particle.

In a further study, guinea pigs were immunized with 10 $\mu$ g of gag p24-equivalent amounts of HIV-like particles

containing two copies of the TMV marker sequence (GAFDTRNRIIEVENGA, SEQ ID No: 21) and boosted with 5µg of the same HIV-like particles at three week intervals. The HIV-like particles were adjuvanted with Freund's complete  
5 adjuvant (and booster doses were adjuvanted with incomplete Freund's adjuvant) or QS21. Sera samples were taken at eleven weeks after the first immunization and tested for reactivity by ELISA with peptides corresponding to the amino acid sequence containing the  
10 V3 neutralizing epitope of HIV-1 MN (TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC, SEQ ID No: 31), the TMV marker sequence (AFDTRNRIIEVEN, SEQ ID No: 1) and p24 (gag) protein produced from a recombinant source. The results are shown in Figure 14 and indicate that the HIV-  
15 like particles containing the TMV marker sequence can generate an immune response to produce antibodies that specifically recognize p24 (gag), gp120 (and specifically the V3 neutralizing epitope) and the TMV marker sequence. The guinea pig antisera were also tested for virus  
20 neutralization using a virus infection inhibition assay as described by Skinner et al., 1988. The end-point titres are shown in Table 2 and indicate that antibodies produced by immunization with the retrovirus-like particles expressing the TMV marker sequence are able to  
25 inhibit cell infection by HIV.

Plasmid pMTHIVMNmHA2-5 was co-transfected with plasmid pSV2neo into Vero cells, and a stable cell line was established that produces HIV-like particles. HIV-like particles were then purified, and guinea pigs  
30 immunized with 10 µg of gag p24-equivalent amounts of HIV-like particles adjuvanted in Freund's complete adjuvant. All animals were boosted three times at 3-week intervals with HIV-like particles adjuvanted in Freund's incomplete adjuvant. Two weeks after the last booster  
35 shots, immune sera were collected and assayed by ELISA for anti-V3 and anti-mHA2 marker reactivities. The

results, presented in Table 1 below, indicate that guinea pigs immunized with HIV-like particles containing the mHA2 marker produced antibodies capable of recognizing peptides representing the mHA2 marker (MHA-1) and V3 loop neutralization domains (CLTB56, CLTB71, and CLTB73).

In a further study guinea pigs were immunized with 10µg of gag p24-equivalent amounts of HIV-like particles containing the mHA2 antigenic marker sequence and boosted three times at three-week intervals with 5µg of the same HIV-like particles. The HIV-like particles were adjuvanted with Freund's complete adjuvant (and booster doses were adjuvanted with incomplete Freund's adjuvant), QS21 or aluminum phosphate (ALUM). Sera samples were taken from the animals eleven weeks after the first immunization and tested for reactivity with peptides corresponding to the amino acid sequence containing the V3 neutralizing epitope of HIV-1<sub>MN</sub> (TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC, SEQ ID No: 31), the mHA2 sequence (GPAKKATLGATFAFDSKEEWCREKKEQWE, SEQ ID No: 22) and p24 (gag) protein produced from a recombinant source. The results are shown in Figure 13 and indicate that the HIV-like particles containing the mHA2 marker sequence can generate antibodies that specifically recognize p24 (gag), gp120 (and specifically the V3 neutralizing epitope) and the mHA2 marker sequence. The guinea pig antisera were also tested for their ability to prevent infection by HIV<sub>MN</sub> as described by Skinner et al., 1988. The end-point titres are shown in Table 2 and indicate that antibodies produced by immunization with the retrovirus-like particles expressing the mHA2 marker sequence are able to inhibit cell infection by HIV.

These data, therefore, demonstrate that the mHA2 marker is immunogenic when presented in the context of an HIV-like particle and that antibodies are also produced against the major neutralizing determinants of the V3 loops from different HIV isolates.

Example 5:

This Example describes the production of HIV-like particles antigenically marked by mutations to an immunodominant region of gp41.

5       The transmembrane domain of gp160 (gp41) contains an immunodominant region containing the amino acid sequence LGIWGCSGKLIC (SEQ ID No: 28) and antibodies recognizing this sequence are present in all HIV-1-infected individuals. Indeed, the detection of antibodies  
10 specific for this immunodominant region is used for clinical diagnosis of HIV-1 infection. Such antibodies specific for this immunodominant region is used for clinical diagnosis of HIV-1 infection. Such antibodies do not generally neutralize HIV-1 however. HIV-like  
15 particles antigenically distinguishable from wild-type HIV-1 were thus produced by mutagenesis of the immunodominant region. Mutants of the immunodominant region that are not recognized by serum from HIV-infection patients were identified by recognition of  
20 peptides containing the mutant sequence with such sera as shown in Table 3. Mutations were introduced by site-specific mutagenesis using a Sculpture™ in vitro mutagenesis system kit manufactured by Amersham Life Science, Amersham International PLC. A SalI BamHI DNA  
25 fragment from p83-19 containing the envelope coding sequence were subcloned into M13 and oligonucleotides containing DNA specifying the mutations shown in Table 3 were used to produced the appropriate mutations to the immunodominant region within gp41 to produce constructs  
30 smIDR, clone 4 and clone 16. Each of the constructs was transfected into VERO cells to produce retrovirus-like particles by immunoblotting. These retrovirus-like particles were analyzed for expression of envelope glycoproteins gp120 and gp160 and gp41. All of these  
35 glycoproteins were present in the various retrovirus-like particles. In particular clone 4 having the amino acid



replacements SGK → AFR in the immunodominant region of gp41 was selected.

#### SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention  
5 provides certain non-infectious, non-replicating,  
retrovirus-like particles and nucleic acid molecules  
encoding them as, for example, immunogenic preparations  
useful for vaccination, the generation of retroviral-  
specific antisera and as antigens in diagnostic methods  
10 and kits. The retrovirus-like particles may have been  
rendered non-infectious by modifications to the pol  
and/or gag gene products. Particular retrovirus-like  
particles contain non-retroviral antigenic markers.  
Modifications are possible within the scope of this  
15 invention.

**TABLE 1.**

The ability of retrovirus-like particles containing an antigenic marker to generate a retroviral-specific immune response and a marker-specific immune response.

PEPTIDE	SEQUENCE	SPECIFICITY	SEQ ID NO.	ELISA IgG TITRES <sup>1</sup>		
				GP542	GP543	GP544
MHA-1	GPAKKATLGATFAFDSEKKEQWE	mHA2 marker	22	500	5,000	2,500
CLTB56	NKRRIHIGPGRAFYTTKN	V3(MN)	23	500	500	2,500
CLTB71	NTRKSIYIGPGRAFHTTGR	V3(SF2)	24	500	2,500	2,500
CLTB73	NTRKRIRIQPGRAFTIGK	V3(HXB2)	25	500	1,000	2,500
Irrelevant	MKKTRFVLNSIALGLSVLSTSFVAQATLPSFVSEQNS	Non-HIV	26	100	100	100

<sup>1</sup> Each guinea pig (GP542, GP543 and GP544) was immunized as described in Example 4.

**TABLE 2**

<b>GUINEA PIG</b>	<b>POSITIVE MARKER</b>	<b>ADJUVANT</b>	<b>ANTI-MN TITER</b>
776	mHA2	Alum	-
777	mHA2	Alum	461
778	mHA2	Alum	279
779	mHA2	QS21	1395
780	mHA2	QS21	625
781	mHA2	QS21	625
1	TMV	FIA	65
2	TMV	FIA	50
3	TMV	FIA	50
4	TMV	QS21	50
5	TMV	QS21	50
6	TMV	QS21	286

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TABLE 3

Construct	Immunodominant region	Amino Acid Sequence	Virus-like particles gp160 gp120	Recognition by serum from HIV+ donors'
p83-19	wild type	AVERYLKDQQLLGIVGCSGKLICTTA	+ +	60/60
smIDR	Conservative replacement	AVERYLKDQQLLGIVGCTGRILCTTA	+ +	4/13
clone 4	Replacement	AVERYLKDQQLLGIVGCAFRILCTTA	+ +	1/60
clone 16	—	AVERYLKDQQLLGIVGCTLELICCTTA	+ +	3/60

Notes:

\* Peptide LGIVGCSGKLI (SEQ ID No: 27) or modified versions thereof incorporating the appropriate amino acid changes were used to screen HIV-1<sup>+</sup> sera. Results are expressed as number of sera recognizing the peptide/total number of samples screened.

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CLAIMS

What we claim is:

1. A non-infectious, immunogenic, retrovirus-like particle comprising, in an assembly, gag, pol and env gene products, wherein at least one modification has been made to the pol and/or gag gene product, to effect at least one of the following:

(a) reduce gag-dependent RNA packaging of the gag gene product;

(b) substantially eliminate reverse transcriptase activity of the pol gene product;

(c) substantially eliminate integrase activity of the pol gene product; and

(d) substantially eliminate RNase H activity of the pol gene product.

2. The retrovirus-like particle of claim 1, wherein the reduction of gag-dependent RNA packaging is effected by replacing or deleting at least one amino acid residue contributing to gag-dependent RNA packaging in the gag gene product.

3. The retrovirus-like particle of claim 2, wherein the at least one amino acid is contained within amino acids Cys<sup>392</sup> to Cys<sup>395</sup> of the gag gene product of HIV-1 LAI isolate or the corresponding region of other retroviral gag gene products.

4. The retrovirus-like particle of claim 3, wherein Cys<sup>392</sup> and/or Cys<sup>395</sup> is replaced by serine.

5. The retrovirus-like particle of claim 4, wherein both Cys<sup>392</sup> and Cys<sup>395</sup> are replaced by serine.

6. The retrovirus-like particle of claim 1, wherein the substantial elimination of reverse transcriptase activity of the pol gene product is effected by deletion of at least a portion thereof contributing to reverse transcriptase activity.

7. The retrovirus-like particle of claim 6, wherein the at least a portion of the pol gene product is contained

between amino acids Pro<sup>168</sup> and Leu<sup>77</sup> of the pol gene product of HIV-LAI isolate or the corresponding region of other retroviral pol gene products.

8. The retrovirus-like particle of claim 1, wherein the substantial elimination of integrase activity of the pol gene product is effected by deletion of at least a portion thereof contributing to integrase activity.

9. The retrovirus-like particle of claim 8, wherein the at least a portion of the pol gene product is contained between amino acids Phe<sup>728</sup> and Asp<sup>1016</sup> of the pol gene product of HIV-LAI isolate or the corresponding region of other retroviral pol gene products.

10. The retrovirus-like particle of claim 1, wherein the substantial elimination of RNase H activity of the pol gene product is effected by deletion of at least a portion thereof contributing to RNase H activity.

11. The retrovirus-like particle of claim 10, wherein the substantial elimination of reverse transcriptase activity, integrase activity and RNase H activity all are substantially eliminated by deleting a portion of the pol gene product corresponding to amino acids Pro<sup>192</sup> to Trp<sup>835</sup> of HIV-1 LAI isolate or the corresponding region of other retroviral pol gene products.

12. The retrovirus-like particle of claim 1 additionally comprising at least one non-retroviral antigenic marker.

13. The retrovirus-like particle of claim 12, wherein the at least one antigenic marker is contained within the gag gene product to form a hybrid gag gene product having the particle-forming characteristics of unmodified gag gene product.

14. The retrovirus-like particle of claim 13, wherein said at least one antigenic marker is inserted into an insertion site of the gag gene product at an antigenically-active insertion site.

15. The retrovirus-like particle of claim 14, wherein said insertion site is located between amino acid

residues 210 and 211 of the gag gene product of the HIV-1 LAI isolate or the corresponding location of other retroviral gag gene products.

16. The retrovirus-like particle of claim 15, wherein said at least one antigenic marker comprises from 1 to 4 tandem copies of the amino acid sequence AFDTRNRIIEVEN or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence AFDTRNRIIEVEN.

17. The retrovirus-like particle of claim 12 wherein said at least one antigenic marker is provided by the absence of an amino acid sequence that corresponds to an epitope of a retroviral protein.

18. The retrovirus-like particle of claim 17 wherein an immunodominant region of the env gene product is modified by substitution or removal of at least one amino acid therefrom to substantially prevent recognition of the immunodominant region in the resulting mutation by sera from retrovirus-infected hosts.

19. The retrovirus-like particle of claim 17 wherein the retrovirus is HIV-1 and the immunodominant region contains the amino acid sequence LGIWGCSGKLIC (SEQ ID No: 27).

20. The retrovirus-like particle of claim 19 wherein the modified amino acid sequence in said mutation is selected from the group consisting of LGIWGCTGRILC (SEQ ID No: 28), LGIWGCAFRILC (SEQ ID No: 29) and LGIWGCTLELIC (SEQ ID No: 30).

21. The retrovirus-like particle of claim 1, wherein said env gene product is a modified env gene product in which endogenous anchoring function has been replaced by a different antigenic anchor sequence operatively connected to the env gene product to anchor said env gene product to the retrovirus-like particle.

22. The retrovirus-like particle of claim 21, wherein said anchor sequence is inserted into an insertion site



of the env gene product adjacent to and upstream of functional cleavage sites of the env gene product.

23. The retrovirus-like particle of claim 22, wherein said insertion site is located between amino acid residues 507 and 508 of the env gene product of the HIV-1 LAI isolate or the corresponding location of other retroviral env gene products.

24. The retrovirus-like particle of claim 23, wherein the anchor sequence includes an amino acid sequence WILWISFAISCFLLCVVCWGSSCGPAKKATLGATFAFDSKEEWCREKKEQWE or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence WILWISFAISCFLLCVVCWGSSCGPAKKATLGATFAFDSKEEWCREKKEQWE.

25. The retrovirus-like particle of claim 23, wherein the anchor sequence includes an amino acid sequence WILWISFAISCFLLCVVLLGFIMW or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence WILWISFAISCFLLCVVLLGFIMW.

26. The retrovirus-like particle of claim 23, wherein the anchor sequence includes an amino acid sequence STVASSLALAIMIAGLSFWMCSNGSLQ or a portion, variation or mutant thereof capable of eliciting antibodies that recognize the sequence STVASSLALAIMIAGLSFWMCSNGSLQ.

27. The retrovirus-like particle of claim 1, wherein the env, pol and gag gene products correspond to the env, pol and gag gene products of a human retrovirus.

28. The retrovirus-like particle of claim 27, wherein the human retrovirus is selected from the group consisting of HIV-1, HIV-2, HTLV-1 and HTLV-2.

29. The retrovirus-like particle of claim 28, wherein the human retrovirus is HIV-1 and the env gene product is an LAI env gene product, an MN env gene product, an env gene product from a primary HIV-1 isolate, or an env gene product antigenically equivalent thereto.

30. The retrovirus-like particle of claim 29 wherein said gag and said pol gene products are derived from an

HIV-1 isolate different from the HIV-1 isolate from which the env gene product is derived.

31. The retrovirus-like particle of claim 30 wherein said env gene product is derived from a primary HIV-1 isolate.

32. A nucleic acid molecule, comprising a modified retroviral genome from which long terminal repeats are absent and containing gag, pol and env genes in their natural genomic arrangement and means for expression operatively connected to said modified retroviral genome for production of gene products in cells to produce non-infectious, immunogenic, retrovirus-like particles comprising an assembly of gag, pol and env gene products, wherein at least one codon in the gag or pol gene has been mutated to effect at least one of the following:

(a) reduce gag-dependent RNA packaging activity of the gag gene product;

(b) substantially eliminate reverse transcriptase activity of the pol gene products;

(c) substantially eliminate integrase activity of the pol gene product; and

(d) substantially eliminate RNase H activity of the pol gene product.

33. The nucleic acid molecule of claim 32 wherein said nucleic acid molecular comprises a DNA molecule containing the characteristic genetic elements present in a SacI (678) to XhoI (8944) fragment of the genome of the HIV-1 LAI isolate.

34. The nucleic acid molecule of claim 32 without a primer binding site and/or an RNA packaging signal.

35. The nucleic acid molecule of claim 32, wherein the reduction of gag-dependent RNA packaging is effected by mutagenesis of a region thereof encoding at least one amino acid contained with a region of the gag gene product corresponding to Cys<sup>392</sup> to Cys<sup>395</sup> of the HIV-1 LAI

isolate, or the corresponding region of other retroviral gene products.

36. The nucleic acid molecule of claim 35, wherein a codon encoding Cys<sup>392</sup> and/or Cys<sup>395</sup> is replaced by a codon encoding serine.

37. The nucleic acid molecule of claim 36, wherein codons encoding both Cys<sup>392</sup> and Cys<sup>395</sup> are replaced by codons encoding serine.

38. The nucleic acid molecule of claim 32, wherein the substantial elimination of reverse transcriptase activity of the pol gene product is effected by deletion of at least a part of the pol gene encoding reverse transcriptase.

39. The nucleic acid molecule of claim 38, wherein the at least a part of the pol gene is contained between nucleotides 2586 and 4265 of the pol gene of HIV-1 isolate LAI or the corresponding region of other retroviral pol genes.

40. The nucleic acid molecule of claim 32, wherein the substantial elimination of integrase activity of the pol gene product is effected by deletion of at least a part of the pol gene encoding integrase.

41. The nucleic acid molecule of claim 40, wherein the at least a part of the pol gene is contained between nucleotides 4266 and 5129 of the pol gene of HIV-1 isolate LAI or the corresponding region of other retroviral pol genes.

42. The nucleic acid molecule of claim 32, wherein the substantial elimination of RNase H activity of the pol gene product is effected by deletion of at least a part of the pol gene encoding RNase H.

43. The nucleic acid molecule of claim 32, wherein said modified retroviral genome includes a segment encoding at least one antigenic marker.

44. The nucleic acid molecule of claim 43 wherein the segment encoding the at least one antigenic marker is

contained within the gag gene to provide a modified gag gene encoding a hybrid gag gene product having the particle-forming characteristics of unmodified gag gene product.

45. The nucleic acid molecule of claim 44, wherein the segment encoding the at least one antigenic marker is inserted into the gag gene at an insertion site to provide the antigenic marker at an antigenically-active insertion site in the hybrid gag gene product.

46. The nucleic acid molecule of claim 45 wherein said insertion site is located at the PstI site at nucleotide 1415 of the gag gene of HIV-1 LAI isolate or the corresponding location of other retroviral gag genes.

47. The nucleic acid molecule of claim 46 wherein the segment encoding the at least one antigenic marker comprises from 1 to 4 copies of a DNA sequence selected from the group consisting of:

(a) 5' GCATTCGACACTAGAAATAGAATAATAGAAGTTGAAAAT 3';

(b) 3' CGTAAGCTGTGATCTTTATCTTATTATCTTCAACTTTTA 5';

and

(c) DNA sequences that hybridize with (a) or (b) under stringent conditions.

48. The DNA molecule of claim 47, wherein DNA sequences in (c) have at least about 90% sequence identity with the sequences of (a) or (b).

49. The nucleic acid molecule of claim 32 wherein said env gene has been modified to provide therein a segment encoding an antigenic anchor marker sequence to anchor the env gene product to the retrovirus-like particle, whereby the modified env gene encodes a modified env gene product in which endogenous anchoring function of env has been replaced by the antigenic anchor marker sequence.

50. The nucleic acid molecule of claim 49 wherein the segment encoding the anchor sequence comprises a DNA sequence selected from the group consisting of:

(a) 5' TGGATCCTGTGGATTCCCTTTGCCATATCATGCTTTTTGCTTTG  
TGTGTTTTGCTGGGGTTCATCATGTGG 3';

(b) 3' ACCTAGGACACCTAAAGGAAACGGTATAGTACGAAAAACGAAA  
CACAACAAAACGACCCCAAGTAGTACACC 5'; and

(c) DNA sequences that hybridize with (a) or (b)  
under stringent conditions.

51. The DNA molecule of claim 50, wherein sequences in  
(c) have at least about 90% sequence identity with  
sequences of (a) or (b).

52. The nucleic acid molecule of claim 49 wherein the  
segment encoding the anchor sequence includes a DNA  
sequence selected from the group consisting of:

(a) 5' TCAACAGTGGCAAGTTCCTTAGCACTGGCAATCAT  
GATAGCTGTGCTATCTTTTTGGATGTGTTCCAATGGGTCATTGCAG 3';

(b) 3' AGTTGTCACCGTTCAAGGGATCGTGACCGTTAGTACTATCGA  
CCAGATAGAAAAACCTACACAAGGTTACCCAGTAACGTC 5'; and

(c) DNA sequences that hybridize with (a) or (b)  
under stringent conditions.

53. The DNA molecule of claim 52, wherein sequences in  
(c) have at least about 90% sequence identity with  
sequences in (a) or (b).

54. The nucleic acid molecule of claim 49 wherein the  
segment encoding the anchor sequence includes a DNA  
sequence selected from the group consisting of:

(a) 5' TGGATCCTGTGGATTTCCTTTGCCATATCATGCTTTTTGC  
TTTGTGTTGTTGCTGGGGTTCATCATGTGGGCCTGCCAAAAGGCAAC  
ATTAGGTGCAACATTTGCATTTGATAGTAAAGAAGAGTGGTGCAGAGAG  
AAAAAAGAGCAGTGGGAA 3';

(b) 3' ACCTAGGACACCTAAAGGAAACGGTATAGTACGAAAAACG  
AAACACAACAAACGACCCCAAGTAGTACACCCGGACGGTTTTTCCGTTG  
TAATCCACGTTGTAAACGTAAACTATCATTTCTTCTCACCACGTCTCTC  
TTTTTCTCGTCACCCTT 5'; and

(c) DNA sequences that hybridize with (a) or (b) under  
stringent conditions.

55. The DNA molecule of claim 54, wherein sequences in (c) have at least about 90% sequence identity with sequences of (a) and (b).
56. The nucleic acid molecule of claim 32 wherein said modified retroviral genome is a modified retroviral genome from a human retrovirus.
57. The nucleic acid molecule of claim 56, wherein the human retrovirus is selected from the group consisting of HIV-1, HIV-2, HTLV-1, and HTLV-2.
58. The nucleic acid molecule of claim 56, wherein the human retrovirus is HIV-1 and the env gene is an LAI env gene, an MN env gene, an env gene from a primary HIV-1 isolate, or an env gene antigenically equivalent thereto.
59. The nucleic acid molecule of claim 58 wherein said gag and said pol genes are derived from an HIV-1 isolate different from the HIV-1 isolate from which the env gene is derived.
60. The nucleic acid molecule of claim 59 wherein said env gene is derived from a primary HIV-1 isolate.
61. An immunogenic composition capable of eliciting a retroviral specific immune response, comprising the retrovirus-like particle of claim 1 or the nucleic acid molecule of claim 32 and a carrier therefor.
62. The immunogenic composition of claim 61 formulated for mucosal or parenteral administration.
63. The immunogenic composition of claim 61 formulated for oral, anal, vaginal, or intranasal administration.
64. The immunogenic composition of claim 61 further comprising at least one other immunogenic and/or immunostimulating material.
65. The immunogenic composition of claim 64, wherein the at least one other immunostimulating material is an adjuvant.
66. The composition of claim 65, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum hydroxide, QS21, Quil A, calcium phosphate,

calcium hydroxide, zinc hydroxide, a glycolipid analog, an octaderyl ester of an amino acid, a muramyl dipeptide, a lipoprotein, and Freund's incomplete adjuvant.

67. A method of immunizing a host to produce a retroviral specific immune response, comprising administering to the host an immunoeffective amount of the immunogenic composition of claim 61.

68. A method of determining the presence of antibodies specifically reacting with retroviral antigens in a sample, comprising the steps of:

(a) contacting the sample with the non-infectious, retrovirus-like particle of claim 1 to produce complexes comprising the non-infectious, retrovirus-like particles and any said antibodies present in the sample specifically reactive therewith; and

(b) determining production of the complexes.

69. A method of determining the presence of retroviral antigens in a sample, comprising the steps of:

(a) immunizing a host with the immunogenic composition of claim 61 to produce retroviral antigen-specific antibodies;

(b) contacting the sample with the retroviral antigen-specific antibodies to produce complexes comprising any retroviral antigens in the sample and retroviral antigen-specific antibodies; and

(c) determining production of the complexes.

70. A diagnostic kit for detecting the presence of retroviral antigens in a sample comprising:

(a) at least one retroviral antigen-specific antibody produced in claim 69;

(b) means for contacting the at least one antibody with the sample to produce a complex comprising any retroviral antigens in the sample and said retroviral antigen-specific antibodies; and

(c) means for determining production of the complex.

1/16

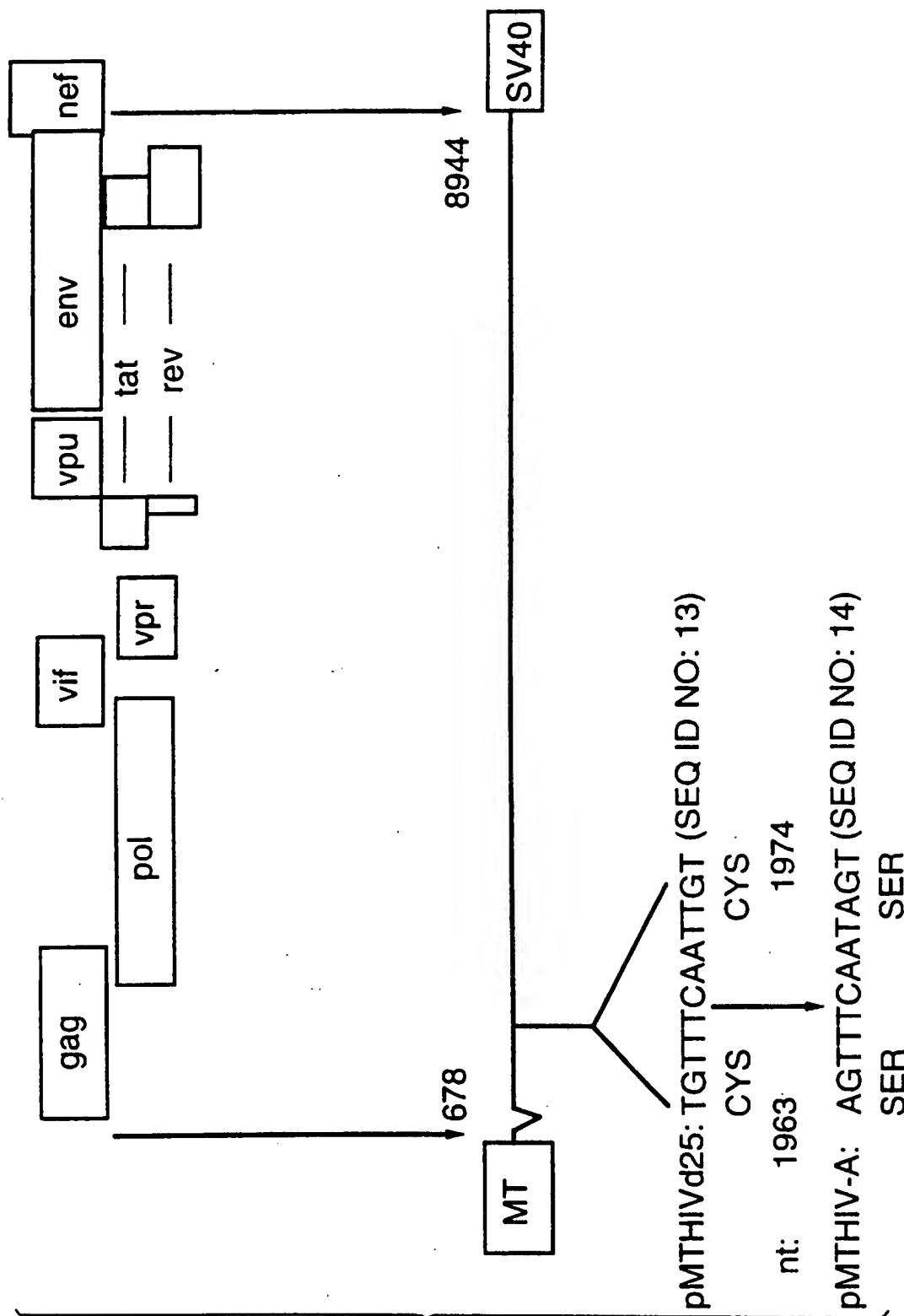


FIG.1



2 / 16

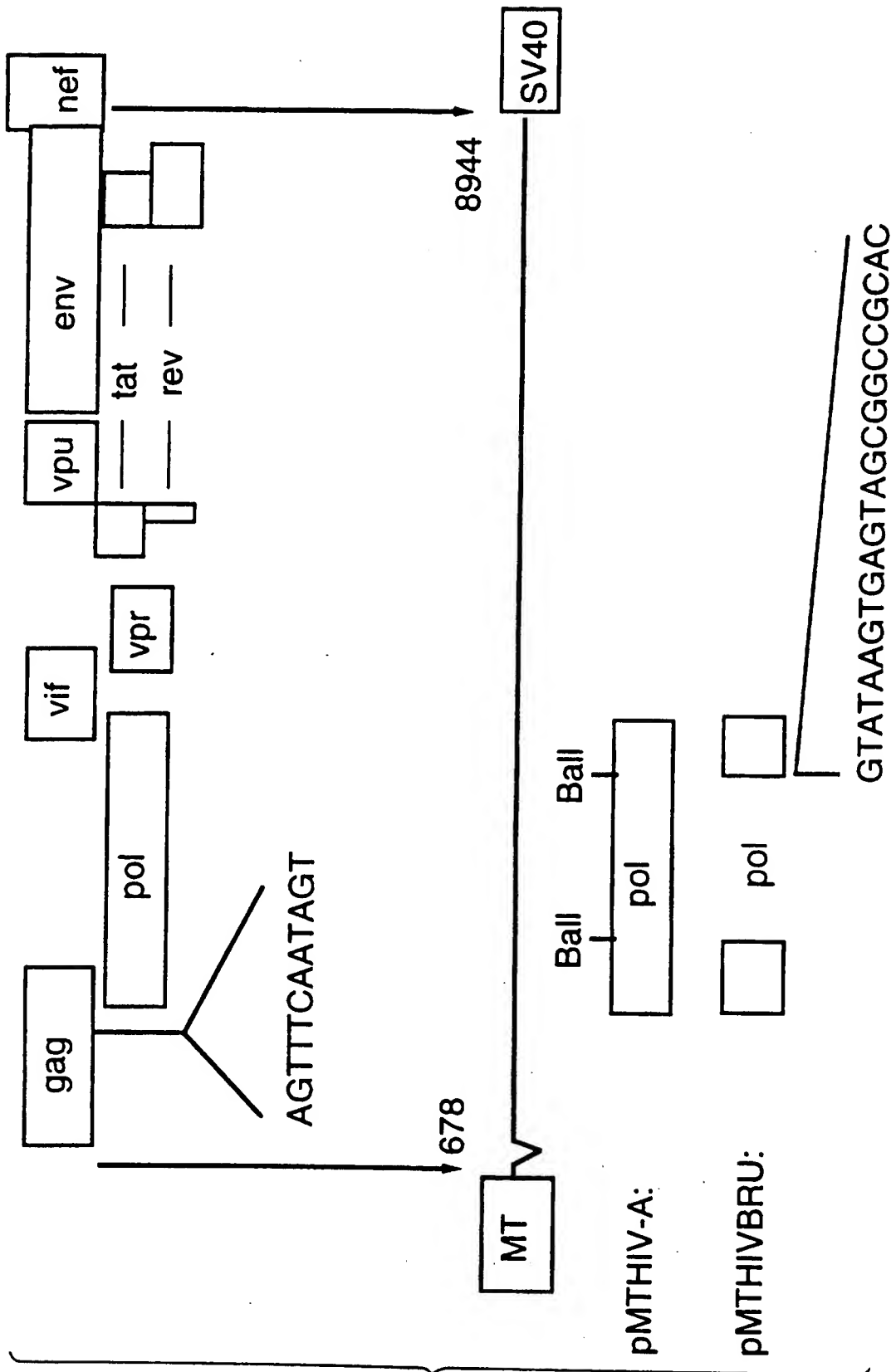


FIG.2

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3/16

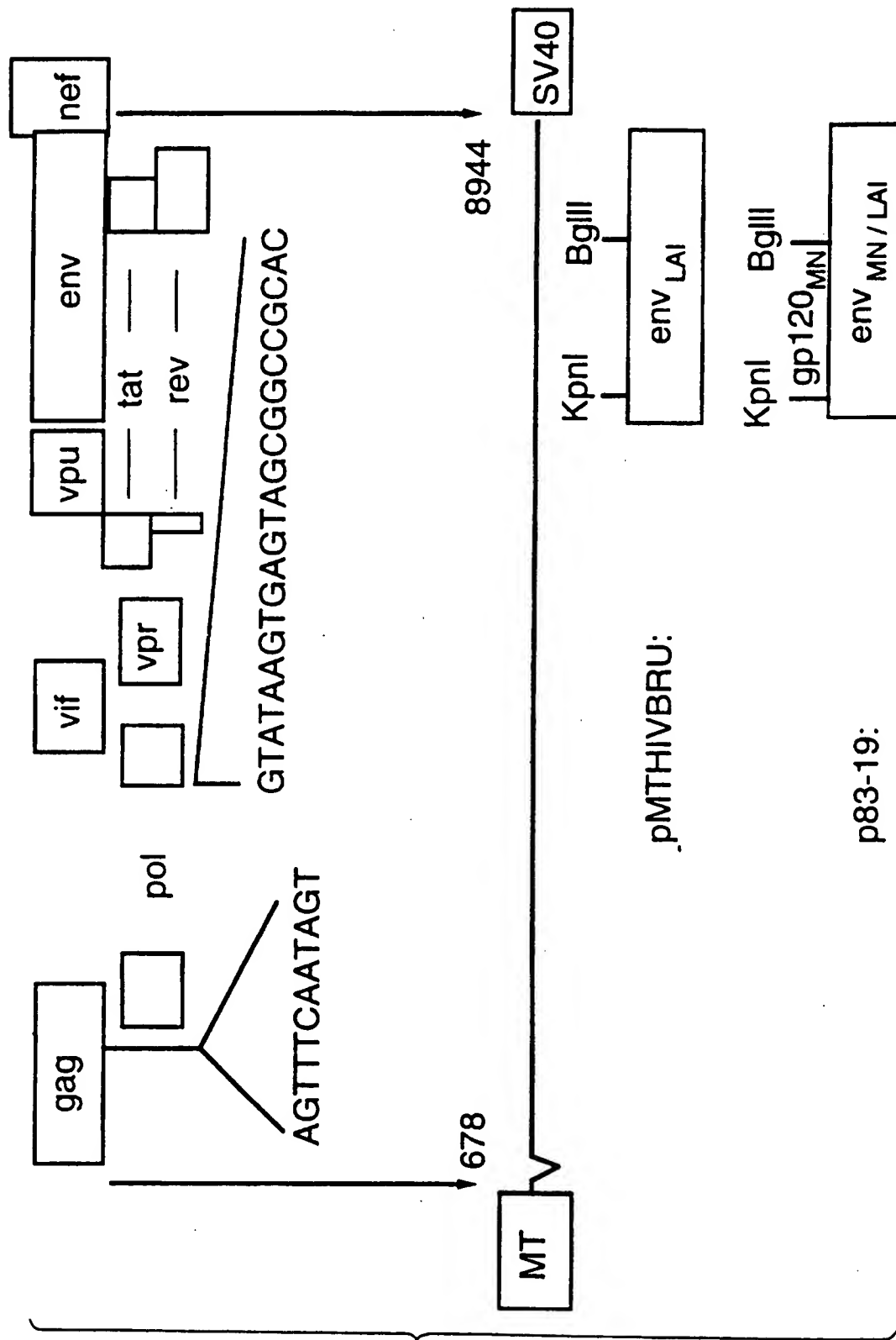


FIG.3

4 / 16

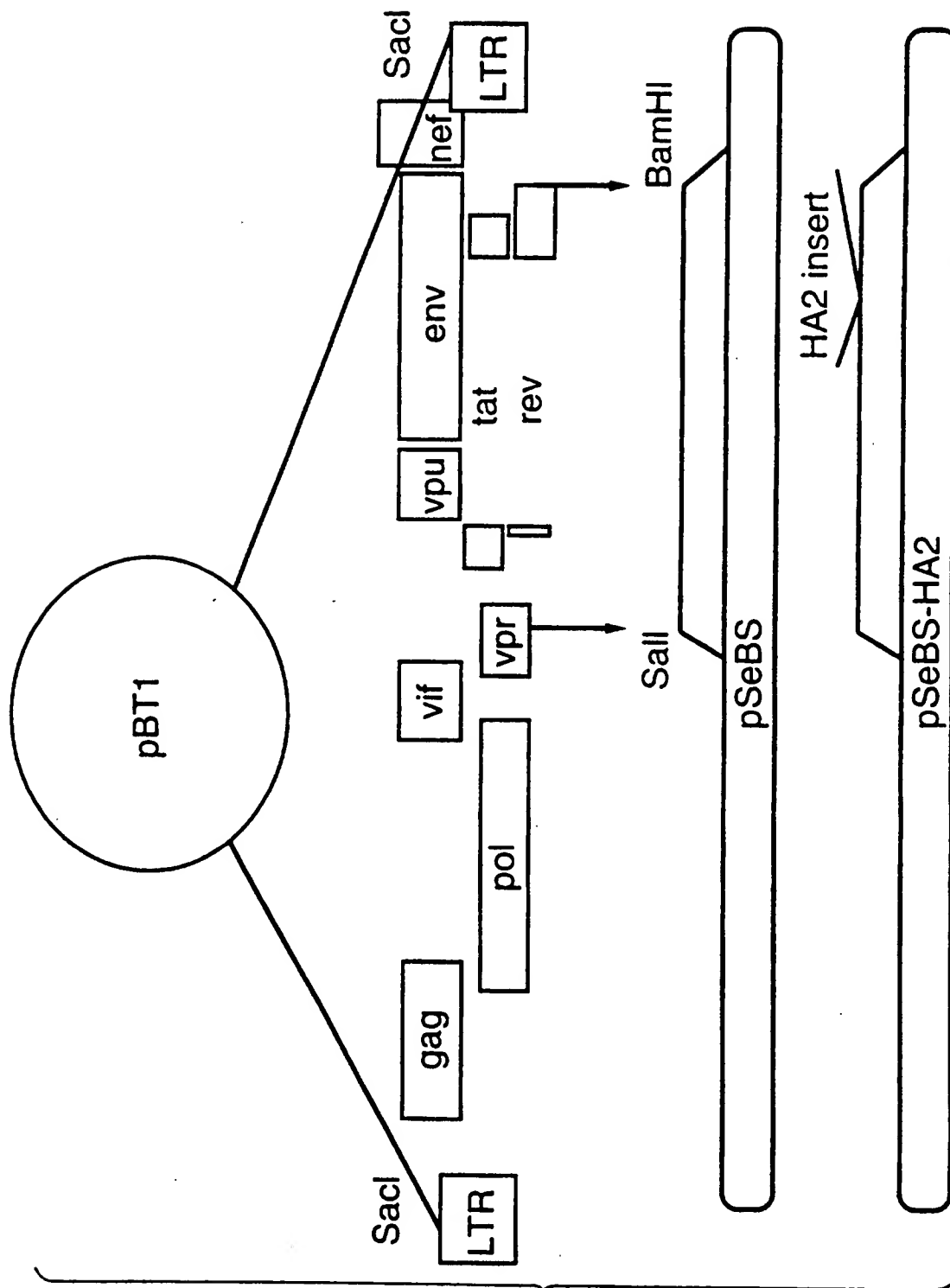
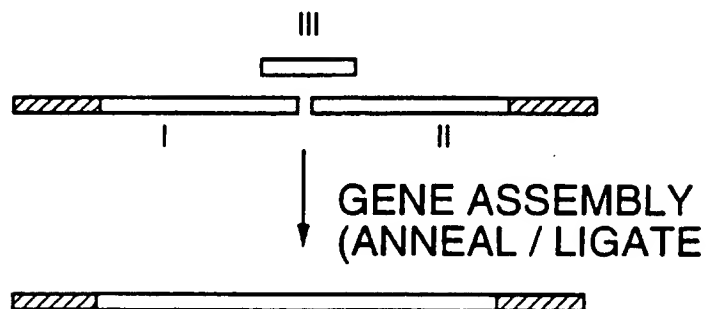


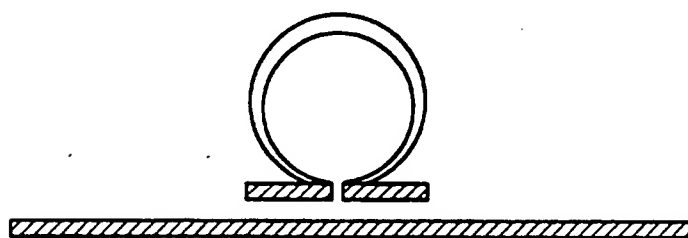
FIG.4

5/16

## GENE ASSEMBLY-AIDED MUTAGENESIS



MUTAGENIC PRIMER



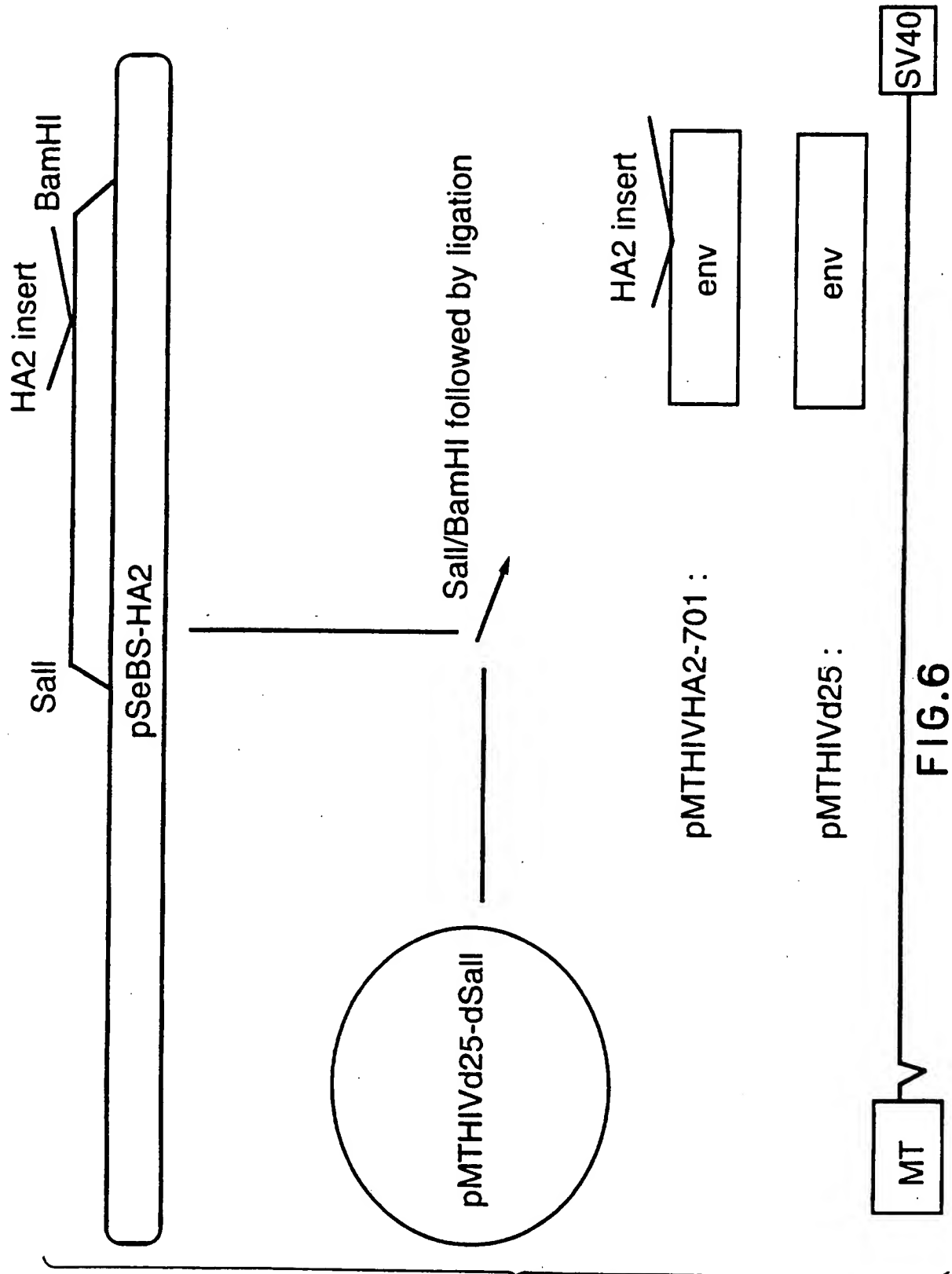
SITE-DIRECTED MUTAGENESIS

 HIV-1 GENE SEQUENCE  
 HA2 GENE SEQUENCE

FIG.5

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6 / 16



# SUBSTITUTE SHEET

7/16

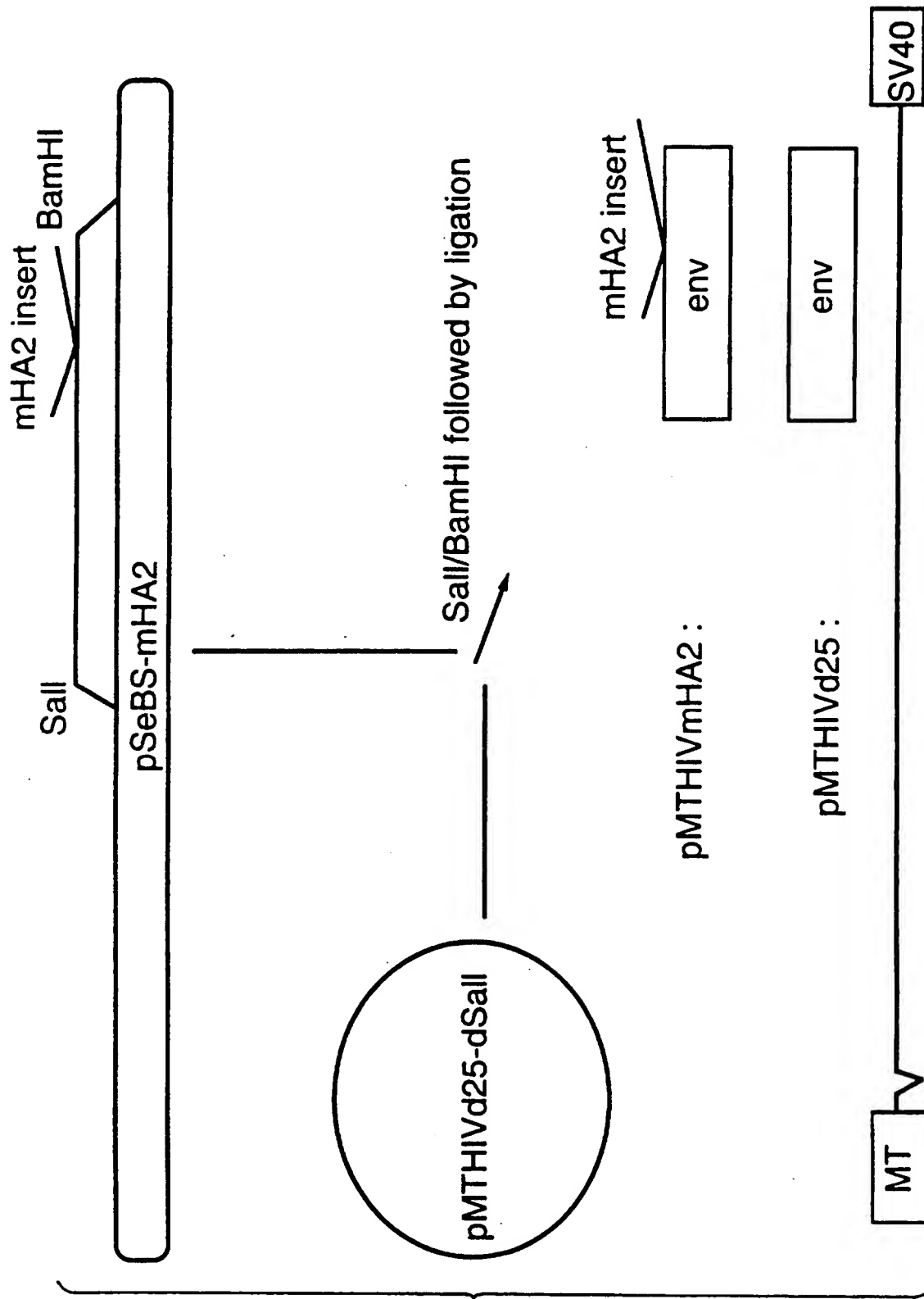


FIG.7

8/16

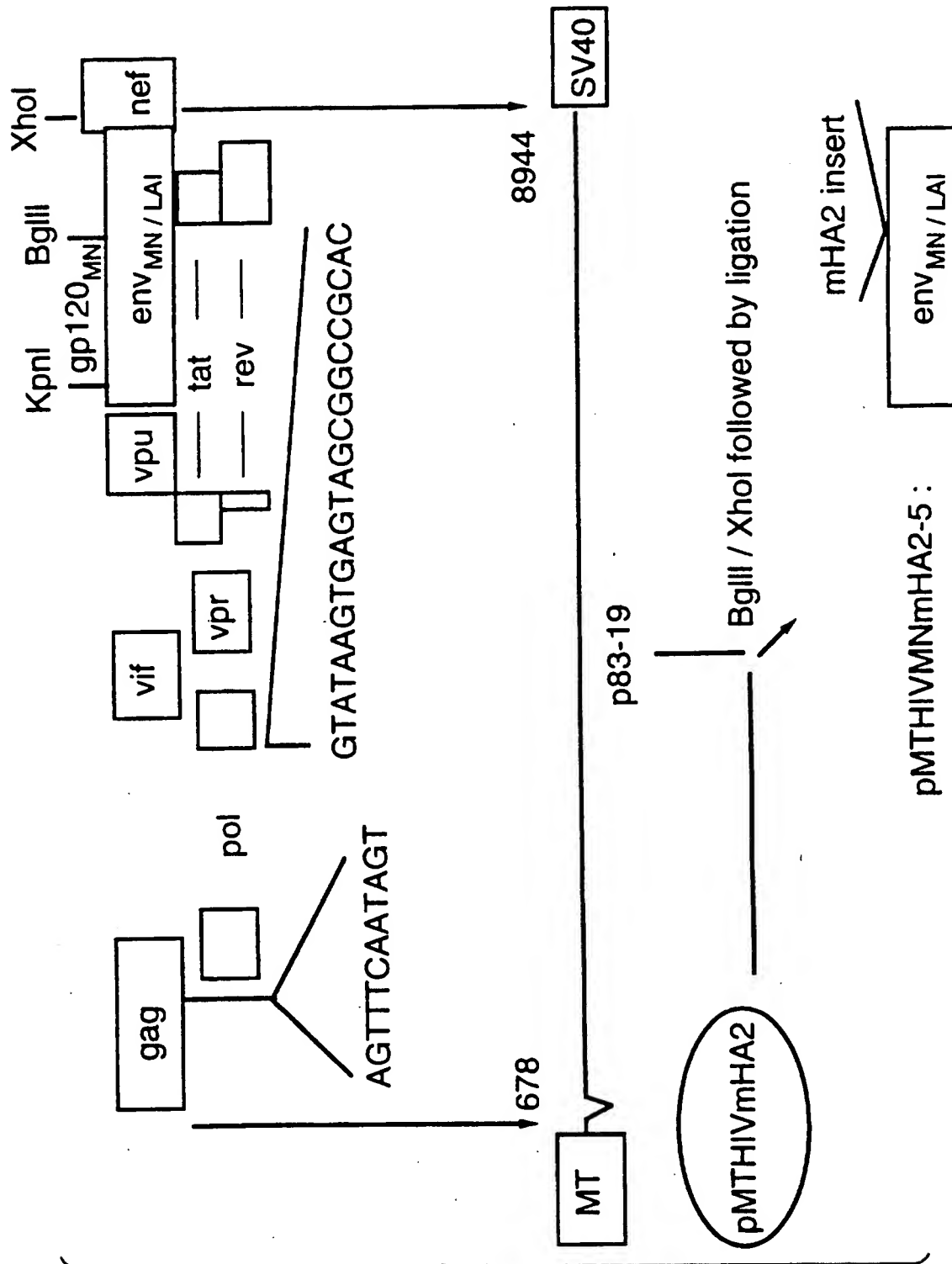


FIG.8

9/16

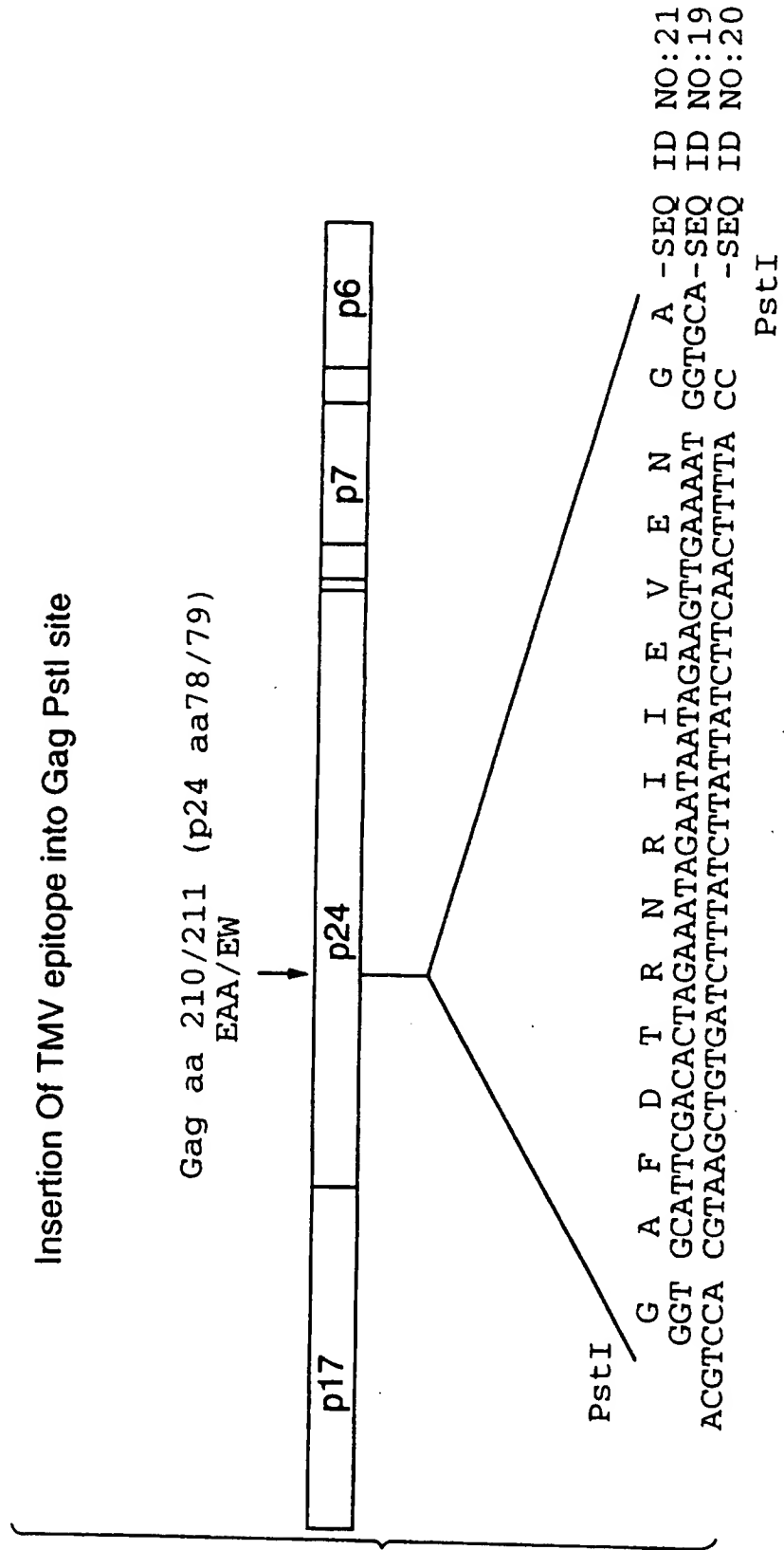
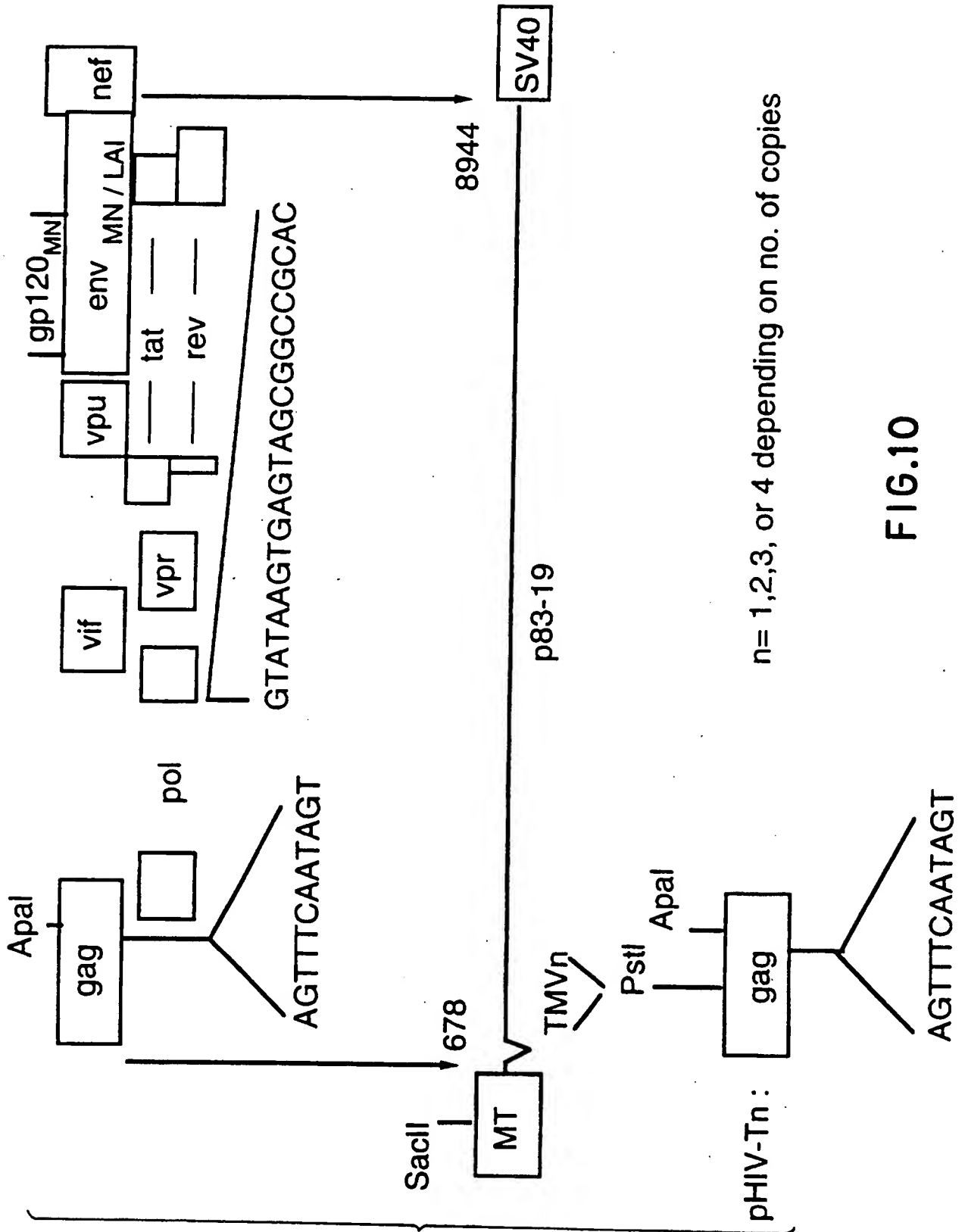


FIG.9

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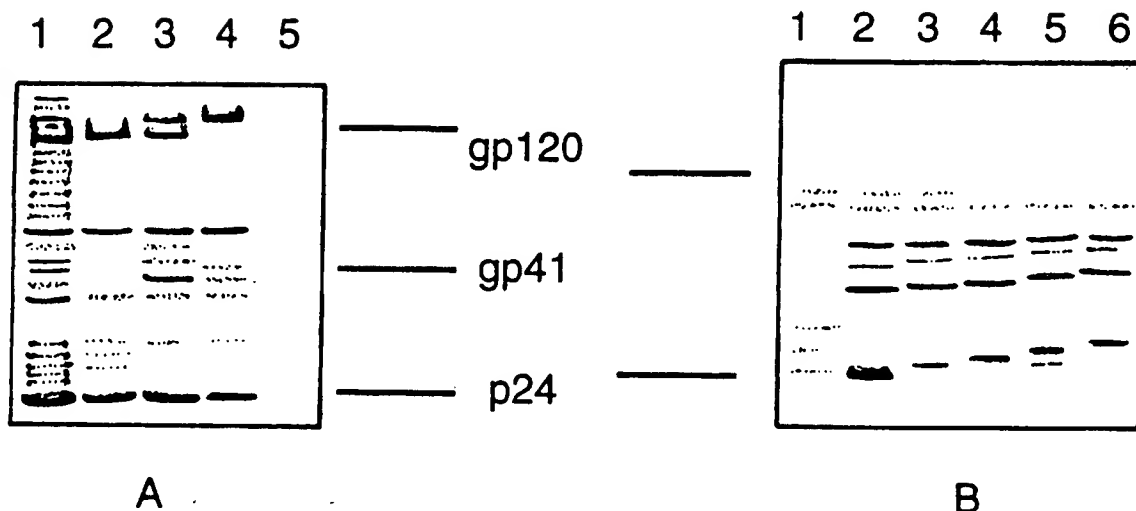
10/16



n= 1,2,3, or 4 depending on no. of copies

FIG.10

11/16

Expression of pseudovirions  
containing positive markers

A. Western blot analysis of pseudovirions containing the human mHA2 epitope (lanes 1 and 2); "wild type" virions (lane 3); pseudovirions containing unprocessed gp160 (lane 4); and pelleted material from mock-transfected Vero cells (lane 5).  
B. Western blot analysis of "wild-type" pseudovirions (lane 2), and pseudovirions containing either one (lane 3), two (lane 4), three (lane 5), or four (lane 6) copies of the TMV epitope.

FIG.11

12/16

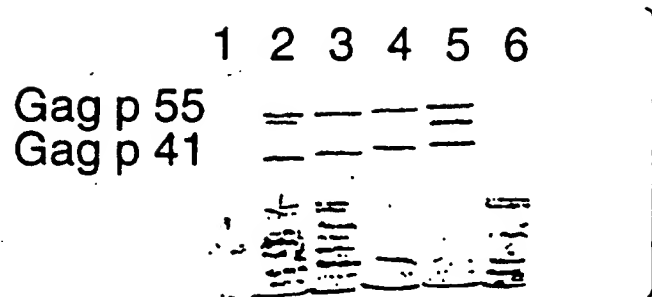
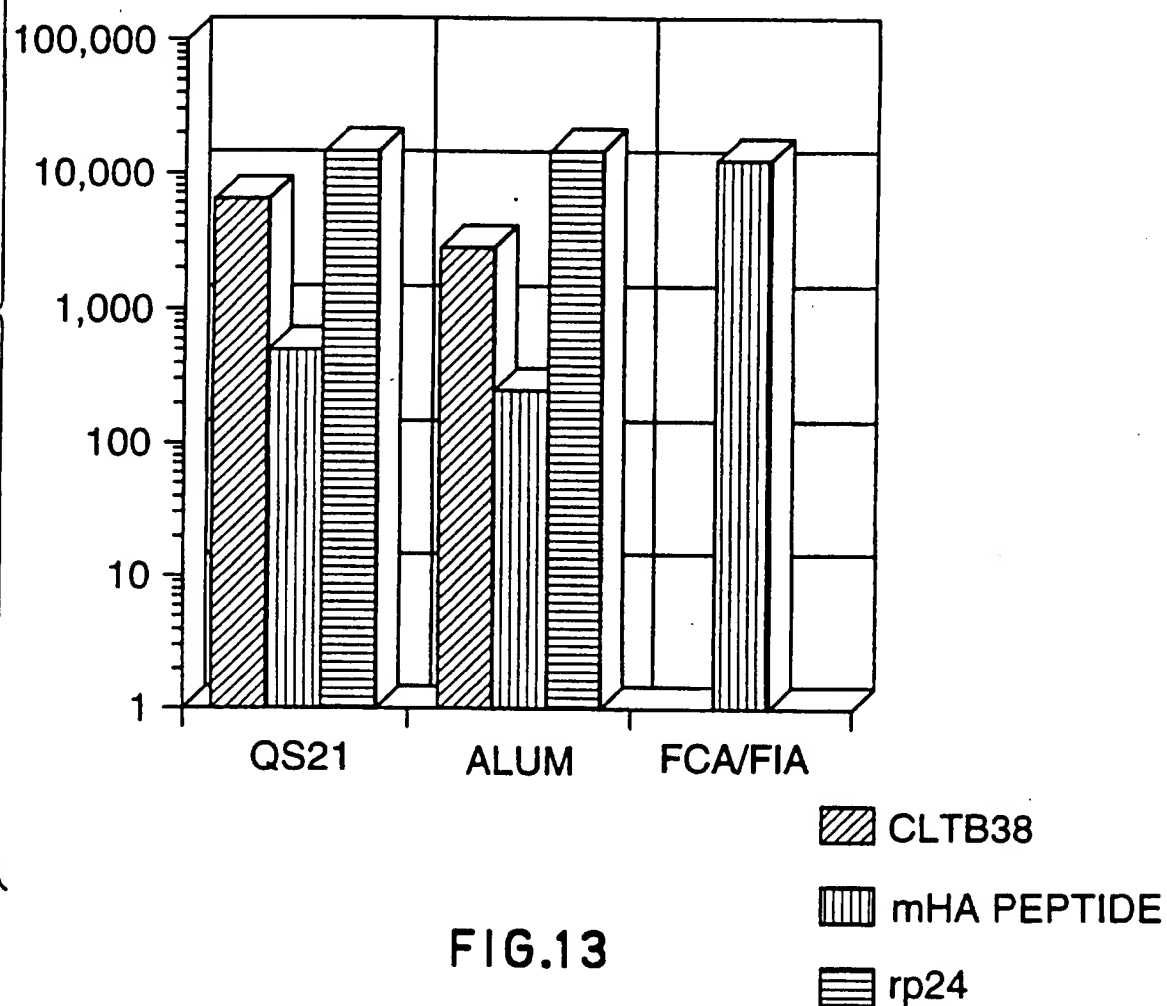


FIG.12

13/16

ANTI-PEPTIDE/PROTEIN IMMUNE RESPONSES TO  
mHA2-CONTAINING VIRIONS

SUBSTITUTE SHEET

14 / 16

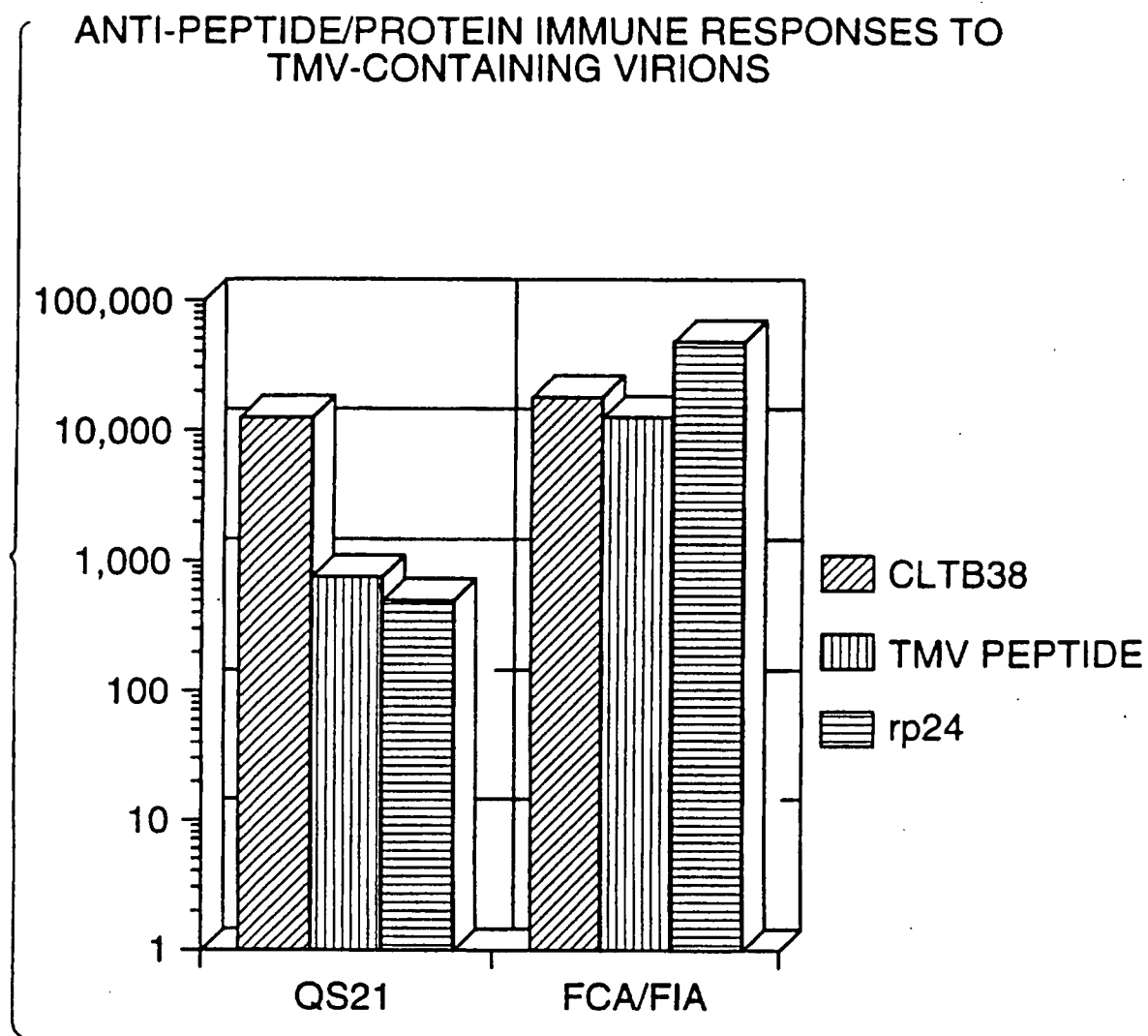


FIG.14

SUBSTITUTE SHEET

15 / 16

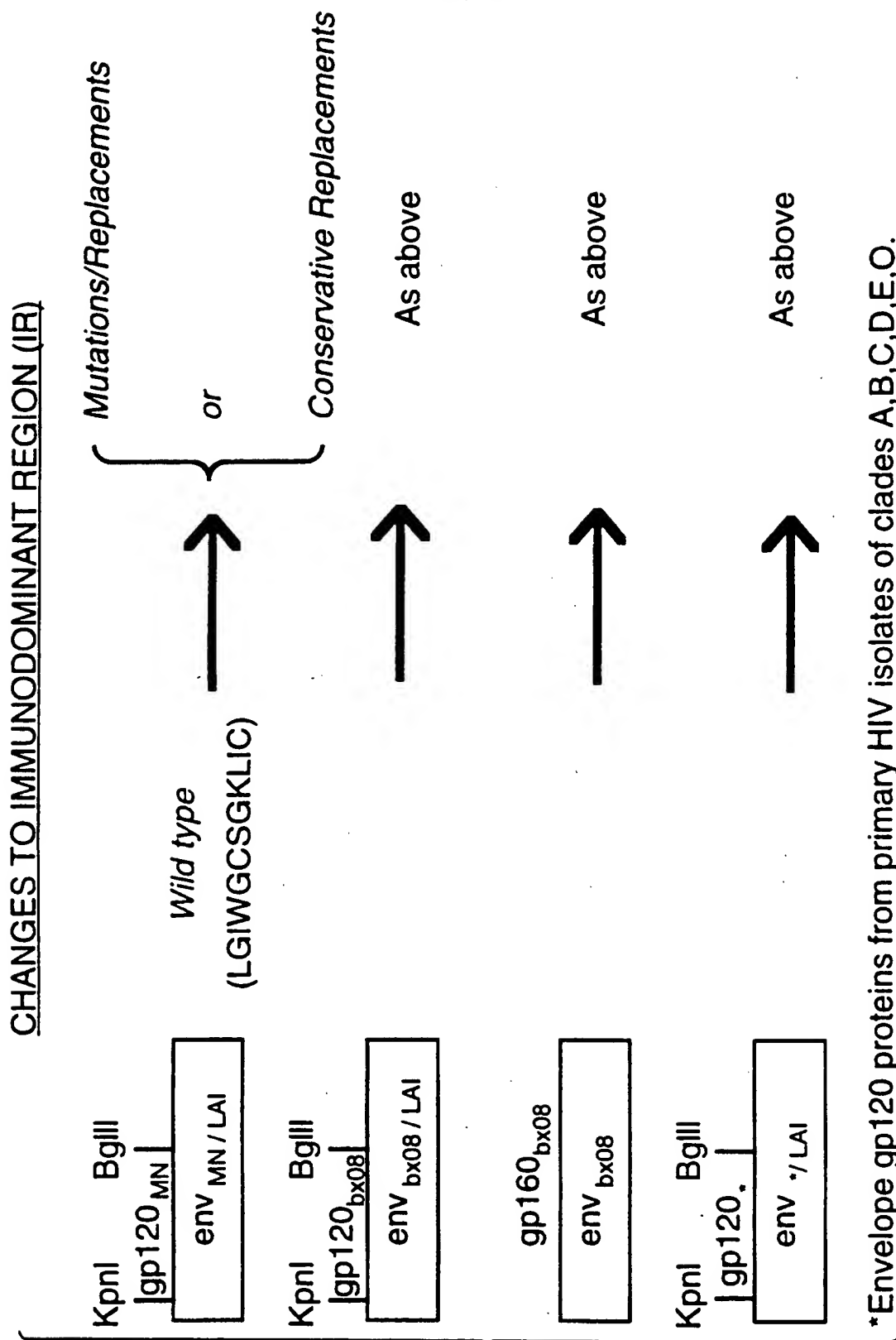
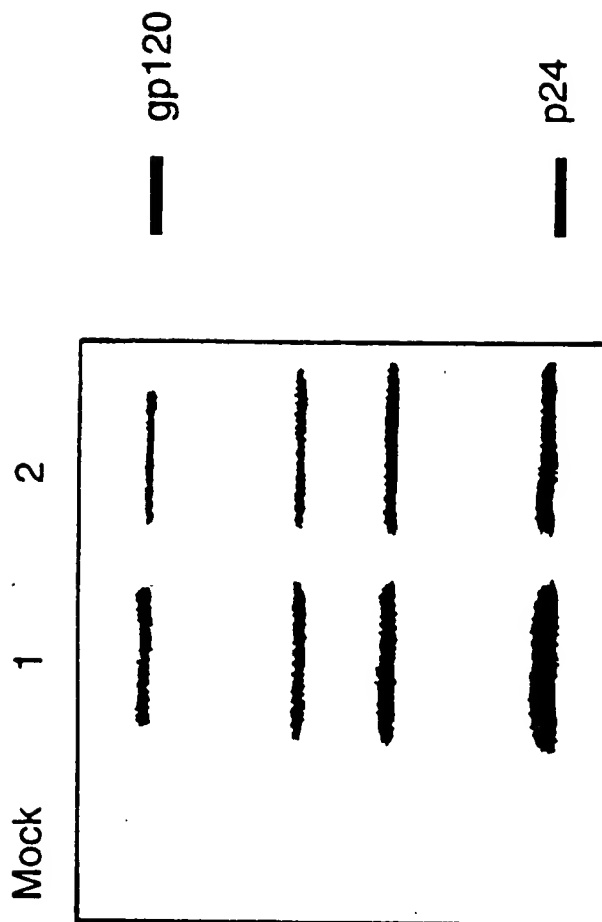


FIG.15

16 / 16

EXPRESSION OF PSEUDOVIRIONS WITH ENVELOPES  
FROM CLINICAL ISOLATE bx08



1 = gp120<sub>bx08</sub>/gp41<sub>lai</sub>  
2 = gp120<sub>bx08</sub>/gp41<sub>bx08</sub>

FIG.16

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N7/00 C12N15/62 C12N15/85 C07K14/08 C07K14/11  
 C07K14/16 C12Q1/68 G01N33/53 G01N33/577 A61K39/12  
 A61K39/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AIDS RESEARCH AND HUMAN RETROVIRUSES, vol. 9, no. sup 1, October 1993, page s33 XP000560222 KLEIN, M. ET AL.: "Development of a cross-neutralizing HIV-1 pseudovirion-based vaccine" see abstract ---	1,8,32, 40
X	WO,A,89 05349 (UNIV AUSTRALIAN ;HAHN JEFFREY ROBERT (AU)) 15 June 1989 see page 5, line 4 - line 13 ---	1,8,32, 40
X	WO,A,93 20220 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 14 October 1993 cited in the application see the whole document ---	1-3
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 May 1996

Date of mailing of the international search report

13. 06. 96

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO,A,91 05860 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 2 May 1991 cited in the application see the whole document ---	1-3
X	WO,A,91 05864 (CONNAUGHT LABORATORIES LIMITED) 2 May 1991 cited in the application see page 8, line 34 - page 9, line 34 see page 14, line 32 - page 17, line 1; claims 1-15; example 15 ---	1,8,32, 40
X	JOURNAL OF VIROLOGY, vol. 64, no. 5, May 1990, pages 1920-1926, XP002003496 ALDOVINI, A. ET YOUNG, R.A.: "Mutations of RNA and protein sequences involved in Human Immunodeficiency Virus type 1 packaging result in production of noninfectious virus" see page 1921, column 2, paragraph 3; figure 1B see page 1924, column 1, paragraph 2 - page 1925, column 1 ---	1-3
A	WO,A,91 07425 (ONCOGEN LIMITED PARTNERSHIP) 30 May 1991 cited in the application see the whole document ---	1,27,61
A	JOURNAL OF VIROLOGY, vol. 66, no. 7, July 1992, pages 4003-4012, XP000560157 ROVINSKI, B. ET AL.: "Expression and characterization of genetically engineered Human Deficiency Virus-like particles containing modified envelope glycoproteins: implications for development of a cross-protective AIDS vaccine" see the whole document ---	18,29,56
A	VIROLOGY, vol. 203, no. 1, 15 August 1994, ORLANDO US, pages 20-28, XP002003497 BOWLES, ET AL.: "Site-directed mutagenesis of the P2 region of the Rous Sarcoma Virus gag gene: effects on gag polyprotein processing" see the whole document ---	1
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,96 05292 (CONNAUGHT LAB ;ROVINSKI BENJAMIN (CA); CAO SHI XIAN (CA); YAO FEI) 22 February 1996 see page 13, line 1 - page 15, line 35 ---	1-6,8, 12-40, 42-69
P,X	JOURNAL OF VIROLOGY, vol. 68, no. 9, September 1994, pages 5969-5981, XP000560252 SAKALIAN, M. ET AL.: "Efficiency and selectivity of RNA packaging by Rous Sarcoma Virus gag deletion mutants" see the whole document ---	1
T	JOURNAL OF VIROLOGY, vol. 69, no. 9, pages 5716-5722, XP002003498 ZHANG, Y. & BARKLIS, E.: "Nucleocapsid protein effects on the specificity of retrovirus RNA encapsidation" see the whole document -----	1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA95/00489

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 2-5, 34-37 and, partially, 1, 12-33, 43-70
- claims 6-11, 38-42 and, partially, 1, 12-33, 43-70

- see cont.-sheet -

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA95/00489

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. claims 2-5,34-37 and, partially, 1,12-33,43-70:

A non-infectious, immunogenic, retrovirus-like particle comprising, in an assembly, gag, pol and env gene products, wherein at least one modification has been made to the gag gene product, to reduce gag-dependent RNA packaging of the gag gene product.

2. claims 6-11,38-42 and, partially, 1,12-33,43-70:

A non-infectious, immunogenic, retrovirus-like particle comprising, in an assembly, gag, pol and env gene products, wherein at least one modification has been made to the pol gene product, to substantially eliminate at least one of the following activities of the pol gene product:

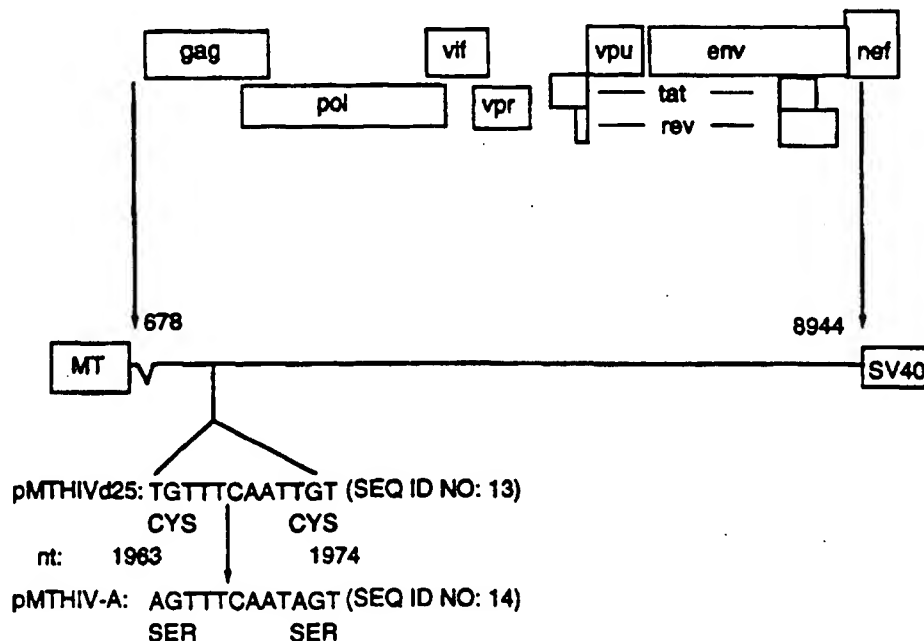
- reverse transcriptase
- integrase
- RNase H



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 7/00, 15/62, 15/85, C07K 14/08, 14/11 14/16, C12Q 1/68, G01N 33/53, 33/577, A61K 39/12, 39/21		A3	(11) International Publication Number: <b>WO 96/06177</b>
			(43) International Publication Date: 29 February 1996 (29.02.96)
(21) International Application Number: PCT/CA95/00489		Michel, H. [CA/CA]; 16 Munro Boulevard, Willowdale, Ontario M2P 1B9 (CA).	
(22) International Filing Date: 22 August 1995 (22.08.95)		(74) Agent: STEWART, Michael, I.; Sim & McBurney, Suite 701, 330 University Avenue, Toronto, Ontario M5G 1R7 (CA).	
(30) Priority Data: 08/292,967 22 August 1994 (22.08.94) US		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/292,967 (CIP) Filed on 22 August 1994 (22.08.94)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant (for all designated States except US): CONNAUGHT LABORATORIES LIMITED [CA/CA]; 1755 Steeles Avenue West, Willowdale, Ontario M2R 3T4 (CA).		(88) Date of publication of the international search report: 25 July 1996 (25.07.96)	
(72) Inventors; and (75) Inventors/Applicants (for US only): ROVINSKI, Benjamin [CA/CA]; 70 Winding Lane, Thornhill, Ontario L4J 5H6 (CA). CAO, Shi-Xian [CA/CA]; Apartment #408, 716 The West Mall, Etobicoke, Ontario M9C 4X6 (CA). YAO, Fei-Long [CN/CA]; 81 Elsa Vineway, North York, Ontario M2J 4H8 (CA). PERSSON, Roy [CA/CA]; 7 Bishop Avenue, Unit 604, North York, Ontario M2M 4J4 (CA). KLEIN,			

(54) Title: RETROVIRUS-LIKE PARTICLES MADE NON-INFECTIOUS BY A PLURALITY OF MUTATIONS



## (57) Abstract

Non-infectious, retrovirus-like particles contain mutations to reduce gag-dependent RNA-packaging of the gag gene product, eliminate reverse transcriptase activity of the pol gene product, eliminate integrase activity of the pol gene product and eliminate RNase H activity of the pol gene product through genetic manipulation of the gag and pol genes. The corresponding nucleic acid molecules are described. The non-infectious, retrovirus-like particles have utility in *in vivo* administration including to humans and in diagnosis.

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